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**Proto-oncogene c-jun expression is induced by  
AML1-ETO in a JNK dependent manner: possible role  
in the pathogenesis of acute myeloid leukemia**

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## ABBREVIATIONS

ACC	animal cell culture
A-E	AML1-ETO
AF-9	MLLT3: mixed-lineage leukemia, translocated to 3 (official gene nomenclature)
AML	acute myeloid leukemia
AP-1	activator protein -1
APL	acute promyelocytic leukemia
APS	ammonium peroxodisulfate
AraC	cytosine arabinoside
Art-1	AML1-regulated transmembrane protein-1
ATF-2	activating transcription factor-2
ATP	adenosin 5' -triphosphate
ATRA	all-trans retinoic acid
BCL-2	B-cell lymphoma-2
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
bZIP	basic domain/leucine zipper
cAMP	adenosine 3', 5' -cyclic monophosphate
CBF	core binding factor
cDNA	complementary DNA
C/EBP $\alpha$	CCAAT/enhancer binding protein alpha
Ci	curie
CML	chronic myeloid leukemia
CMV	cytomegalovirus
comp	competitor
CRE	cyclic-AMP response element
CREB	cyclic-AMP response element binding protein
dAP-1	distal AP-1
ddH <sub>2</sub> O	double distilled water
DMEM	Dulbeccos modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	3' -deoxyribonucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	dithiothreitol
EDTA	ethylendiaminetetraacetatic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular regulated kinase
ETO	eight-twenty-one

FAB	French-American-British
FBS	fetal bovine serum
fg	femtogram
FITC	fluorescein isothiocyanate
G418	genticin disulfate salt
G6PD	glucose-6-phosphate dehydrogenase
G-CSF(R)	granulocyte-colony stimulating factor (receptor)
GM-CSF	granulocyte/macrophage-colony stimulating factor
GST	glutathione S-transferase
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
Ig	immunoglobulin
IL	interleukin
inv(16)	inversion 16
JIP-1	c-Jun interacting protein-1
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
kDa	kilodalton
LB	<i>luria bertani</i>
MAP(K)	mitogen activated protein (kinase)
M-CSF	macrophage-colony stimulating factor
MEF-2	myocyte enhancer factor-2
MgCl <sub>2</sub>	magnesium chloride
MLL	mixed lineage leukemia
mRNA	messenger RNA
n	number of patients
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-jun	nuclear factor jun
NF-κB	nuclear factor-kappaB
NHR	nervy homology region
NP40	octylphenylpolyethylene glycol
PAGE	polyacrylamide gel electrophoresis
pAP-1	proximal AP-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	<i>pondus hydrogenii</i>
PML	promyelocytic leukemia protein
PMSF	phenylmethylsulphonyl fluoride
RARα	retinoic acid receptor alpha
RNA	ribonucleic acid
rpm	revolutions per minute
s	shift

ss	supershift
SAPK	stress-activated protein kinase
S.D.	standard deviation
SDS	sodium dodecyl sulfate
Ser	serine
SMMHC	smooth muscle myosin heavy chain
SP-1	specificity protein-1
STAT	signal transducers and activators of transcription
t(8;21)	translocation(8;21)
TAE	Tris/acetate/EDTA
TBE	Tris/borate/EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween20
TCR	T-cell receptor
TE	Tris/EDTA
TEMED	N, N, N', N'-tetramethylenediamine
Tet	tetracycline
TGFβ	transforming growth factor beta
Thr	threonine
TLE	transducin-like enhancer
TPA	12-o-tetradecanoylphorbol 13-acetat
Tris	tris(hydroxymethyl)aminomethan
Tris·Cl	Tris hydrochloride
TWEEN20	polyoxyethylenesorbitan monolaurate
Tyr	tyrosine
U	unit
U937Z/A-E	U937 cell line with Zn <sup>2+</sup> -inducible expression of AML1-ETO
U937T/A-E	U937 cell line with Tet-regulated expression of AML1-ETO
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
Zn <sup>2+</sup>	zinc

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## 1. INTRODUCTION

### 1.1 Proto-oncogene c-jun and JNK signaling pathway

The nuclear proto-oncogene c-jun encodes a major component of the AP-1 (activator protein-1) transcription factor family comprising the Jun (c-Jun, JunB, JunD), Fos (c-Fos, Fra-1, Fra-2, FosB) and CREB/ATF (ATF-2, ATF-3) proteins.<sup>1,2</sup> AP-1 transcription factors belong to the bZIP superfamily of proteins, which dimerize and bind DNA via the basic domain/leucine zipper region, respectively.<sup>3-5</sup> Dimerization is a prerequisite for binding to the DNA consensus site in target gene promoters and thus for transcriptional activity.<sup>3,6</sup>

c-Jun homodimerizes or heterodimerizes with other AP-1 family members and dependent on the heterodimerizing partner, c-Jun recognizes either TPA or cAMP response elements in promoters.<sup>7-9</sup> TPA response elements are phorbol ester and growth factor inducible and preferentially bind c-Jun homodimers or c-Jun/c-Fos heterodimers. cAMP response elements (CRE) preferentially bind ATF/CREB family members or ATF-2/c-Jun heterodimers.<sup>10</sup>

Interestingly, the promoter region of the c-jun gene itself contains two variant AP-1/CRE binding sites, a proximal and a distal site, which are important for basal promoter activity.<sup>11</sup> The more proximal AP-1 site differs from the AP-1 site in the collagenase promoter by a nucleotide insertion (underlined) and has the sequence: 5'-TGACATCA-3'. The distal AP-1 site differs from the AP-1 site in the SV40 enhancer by an additional base pair (underlined): 5'-TTACCTCA-3'.<sup>11</sup> c-Jun preferentially heterodimerizes with ATF-2 at these two AP-1/CRE sites.<sup>12-14</sup> The more proximal AP-1/CRE (pAP-1) site is the main site at which c-Jun stimulates its own transcription in a positive autoregulatory loop.<sup>15,16</sup>



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For transcriptional and autoregulatory activity, c-Jun requires phosphorylation by the Jun NH<sub>2</sub>-terminal kinase (JNK).<sup>17-19</sup> JNK belongs together with p38 and ERK to the mitogen activated protein (MAP) kinase family. MAP kinases (MAPK) are phosphorylated on threonine and tyrosine residues by dual-specificity MAPK kinases, which are themselves activated by MAPK kinase kinases.<sup>20</sup> JNK interacts with c-Jun via a small region (delta domain) within the NH<sub>2</sub>-terminal activation domain<sup>19,21</sup> and phosphorylates Serine 63 (Ser63) and Serine 73 (Ser73) in c-Jun, which increases the transactivation potential of c-Jun.<sup>17,18</sup> After phosphorylation, inactive JNK remains bound to c-Jun and mediates degradation of c-Jun by ubiquitination.<sup>22</sup>

c-Jun is an environmental sensor and the JNK signaling pathway connects c-Jun to a variety of extracellular factors, such as UV irradiation,<sup>12,23</sup> growth factors<sup>24</sup> and cytokines.<sup>25-27</sup> Thus, c-Jun and JNK regulate crucial physiological processes like proliferation, apoptosis or differentiation. Functional properties of c-Jun and JNK vary dependent on the cell type and cell stage, the incoming signals and also the dimer composition.<sup>28,29</sup>

c-Jun is, however, also located at the end of signaling cascades that include important oncogenes active in human tumors.<sup>1,13,30</sup> Oncogenes like Ha-ras,<sup>31</sup> cot<sup>32</sup> and met<sup>33</sup> require phosphorylation of Ser63 and Ser73 in c-Jun by JNK for their transformation capacities. c-Jun alone is, in contrast to its viral counterpart v-Jun, a relatively weak inducer to transformation.<sup>34</sup> It has been described that N-terminal phosphorylation of Ser63 and Ser73, located in the transactivation domain of c-Jun, augments c-Jun mediated transformation.<sup>35,36</sup> This is further indicated in transgenic mice, which express a dominant negative form of c-Jun lacking the transactivation domain including the Ser63 and Ser73 phosphorylation sites. These mice are protected against skin tumor promotion.<sup>37</sup>

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Furthermore, overexpression of c-Jun has been associated with transformation and tumor promotion. Overexpression of c-Jun can result from sustained JNK phosphorylation, which prevents degradation of c-Jun by ubiquitination.<sup>38,39</sup> Overexpression of c-Jun and constitutive activation of JNK have therefore been associated with a variety of solid cancers.<sup>40-42</sup> Generally, reversion of highly tumorigenic cell lines to a non-tumorigenic phenotype is associated with c-Jun downexpression.<sup>43</sup>

In addition, overexpression of c-Jun and sustained JNK activation has been shown to play an important role in leukemic cells with translocations. Chronic myeloid leukemia (CML) cells with the translocations t(9;22) or t(3;21) show elevated AP-1 activity, which has been associated with the leukemic transformation process.<sup>44-47</sup> Studies on t(9;22) have further revealed that the resulting BCR-ABL fusion gene constitutively activates the JNK signaling pathway and increases c-Jun expression in BCR-ABL positive patient cells.<sup>48</sup>

There are indications that enhanced AP-1 activity might also play a role in translocations associated with acute myeloid leukemia (AML). Overexpression of the AML1-ETO fusion gene, resulting from the translocation t(8;21), in fibroblast cells, results in transformation and has been associated with increased levels of phosphorylated Ser63 in c-Jun and enhanced AP-1 activity.<sup>49</sup> The pathophysiological importance of these findings, however, has not been confirmed in primary leukemia patient samples on the basis of increased levels of c-Jun expression.

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## 1.2 Acute myeloid leukemia and translocation t(8;21)

Acute myeloid leukemia (AML) accounts for about 1-1.5% of all cancers in Western countries.<sup>50</sup> AML is a clonal disorder resulting from an acquired somatic mutation in hematopoietic progenitor cells and is characterized by an accumulation of immature myeloid cells in the bone marrow, which are arrested in maturation. Frequently in AML, the acquired mutation is the consequence of a balanced reciprocal chromosomal translocation, which disrupts genes residing in the breakpoint regions of the translocation by generating fusion genes.<sup>51,52</sup> The genes located at these breakpoints often encode transcription factors which are master regulators of hematopoietic cell differentiation, apoptosis or proliferation.<sup>53</sup>

Despite the heterogeneity of AML, the identification of recurring chromosomal rearrangements and the resulting molecular abnormalities have identified distinct subgroups of patients with predictable clinical features and therapeutic responses.<sup>54</sup> The most frequent cytogenetic abnormalities in AML, accounting for approximately 40% of all AMLs, are the translocations t(8;21), t(15;17), inv(16) or t(9;11), encoding for the AML1-ETO, PML-RAR $\alpha$ , CBF $\beta$ -SMMHC or MLL-AF9 fusion proteins, respectively.<sup>51</sup>

AML1-ETO is associated with approximately 40% of AML cases with the M2 subtype according to the French-American-British (FAB) classification and represents one of the most frequent chromosomal translocation in AML (18-20%).<sup>51</sup> t(8;21) is a balanced translocation between chromosomes 8 and 21 resulting at the molecular level in the fusion of the AML1 gene normally located on chromosome 21q22 with the ETO (Eight-Twenty-One) gene on chromosome 8q22.<sup>55,56</sup>

The wildtype AML1 gene encodes for the CBF $\alpha$ 2 protein, which is a physiological component of the core binding factor (CBF) family.<sup>57</sup> AML1 appears to function as a transcriptional organizer necessary for the development of definitive hematopoietic stem cells.<sup>58</sup> In fact, CBF null mice die *in utero* in the absence of terminal hematopoietic differentiation.<sup>59-61</sup>

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Transcriptional function of AML1 is mediated through the core enhancer DNA sequence present in promoters and enhancers of a large number of hematopoietic specific genes.<sup>62</sup> AML1 binds this sequence through the *runt* homology domain and its DNA binding affinity is increased by heterodimerization with CBF $\beta$ .<sup>63</sup> Finally, AML1 has been shown to co-operate with other basic transcription factors in activating a set of hematopoietic specific genes.<sup>64</sup>

ETO was unknown prior to its identification as the fusion partner of AML1 in t(8;21).<sup>55,65,66</sup> ETO is expressed as a nuclear phosphoprotein in brain and in CD34+ hematopoietic cells.<sup>67,68</sup> It contains four evolutionarily conserved regions, the so-called runty homology regions (NHR) 1-4, which have been shown to make several contacts with co-repressors and histone deacetylases.<sup>69</sup> Although ETO is a nuclear zinc-finger containing protein and thus might function as a transcriptional regulator, there is no experimental evidence to suggest that it can directly bind to DNA.

In the translocation t(8;21), the DNA binding domain (*runt* domain) of AML1 is fused to nearly the complete ETO gene yielding a protein of 752 amino acids.<sup>55,65,70</sup> Several important regions of AML1 are lost, like the C-terminal transactivation domain, interaction sites for TLE and sin3 co-repressors, the nuclear targeting signal and a MAPK phosphorylation site.<sup>71</sup> AML1-ETO sequesters the co-activator CBF $\beta$  from wildtype AML1 by binding CBF $\beta$  through the retained *runt* domain more efficiently.<sup>72</sup> Thus, AML1-ETO can bind to AML1 target genes instead of wildtype AML1,<sup>62</sup> and dominantly represses transcription of AML1 target genes by permanently tethering a repressor complex to AML1 responsive promoters through ETO.<sup>73-76</sup> The importance of disruption of wildtype AML1 by AML1-ETO is shown by a similar phenotype in AML1 knock-out and AML1-ETO knock-in studies.<sup>77,78</sup>

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AML1-ETO blocks the transactivation of wildtype AML1 target genes important for myeloid differentiation, like the TCR $\beta$  reporter, the GM-CSF promoter, c-fos,<sup>79</sup> Art-1,<sup>80</sup> or the TGF $\beta$  signaling pathway.<sup>81</sup> Furthermore, AML1-ETO disrupts the protein-protein interactions of AML1 with important myeloid transcription factors like C/EBP $\alpha$ <sup>82,83</sup> or MEF-2,<sup>84</sup> and also represses transactivation through those transcription factors. Therefore, AML1-ETO might be responsible for the differentiation arrest in t(8;21). Along with that, several cellular and murine model systems have documented the ability of AML1-ETO to inhibit myeloid differentiation.<sup>45,85-88</sup>

It has recently been shown that AML1-ETO might also be responsible for the gain of function properties characterizing leukemogenesis. Expression of AML1-ETO in primary human CD34+ stem cells, the target cell affected in AML, results in a proliferative and survival advantage of leukemic cells, which promotes their expansion.<sup>89</sup> Introduction of the AML1-ETO cDNA into the AML1 locus by homologous recombination leads to embryonic lethality in heterozygous animals.<sup>77,78</sup> Culturing the yolk sac cells of these mice, however, yields dysplastic monocytic colonies.<sup>77</sup> In a similar AML1-ETO knock-in mouse study, aberrant myelomonocytic colonies are derived from fetal liver.<sup>78</sup> Therefore, despite the fact that AML-ETO alone has not been shown to cause leukemia,<sup>90,91</sup> these studies indicate that AML1-ETO encourages uncontrolled cell growth and might predispose to leukemia.

Along with that, several positively regulated target genes of AML1-ETO have been described. AML1-ETO has been reported to activate the macrophage-colony stimulating factor (M-CSF) receptor promoter in co-operation with wildtype AML1 via the core enhancer consensus site. It is proposed that AML1-ETO enhances the transactivation potential of wildtype AML1 by sequestering AML1 bound corepressors.<sup>92</sup>

Furthermore, AML1-ETO has been shown to transactivate the BCL-2 promoter via the AML1 consensus site in AML1-ETO expressing Kasumi-1 cells.<sup>93</sup> The physiological relevance of these findings is, however, doubtful, since in primary t(8;21) positive patient cells, BCL-2 expression is lower than in other forms of leukemia.<sup>94,95</sup>

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It has recently been shown that expression levels of the granulocyte-colony stimulating factor (G-CSF) receptor are increased in AML1-ETO containing cell lines and in fresh t(8;21) positive leukemic cells.<sup>96</sup> The data indicates that AML1-ETO induces the expression of the G-CSF receptor promoter in an indirect manner by increasing the expression of C/EBP $\epsilon$ , which transactivates the G-CSF receptor promoter.<sup>96</sup>

Interestingly, a differential display analysis using AML1-ETO expressing cells has revealed a surprising number of genes that are increased in their expression levels, but are not target genes of AML1.<sup>97</sup>

Only few of these positively regulated target genes of AML1-ETO have been confirmed in primary t(8;21) positive cells. Furthermore, the molecular mechanisms behind the gain of function properties of AML1-ETO remain largely unknown.

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## 2. AIM OF THE STUDY

- Based on the role of c-Jun and the JNK signaling pathway in the transformation process of chromosomal translocations associated with CML, we hypothesized that c-Jun might also play a role in reciprocal chromosomal translocations in AML.

What are the expression levels of c-jun in primary AML patient cells with the most common AML-associated cytogenetic abnormalities: t(8;21), t(15;17), inv(16) or t(11q23/MLL)?

- It has previously been described that overexpression of AML1-ETO in fibroblast cells results in an increase in phosphorylated Ser63 in c-Jun, which has been implicated in the transformation process.<sup>49</sup> Phosphorylated c-Jun can autoregulate its expression.<sup>15,16</sup>

Are the expression levels of c-jun enhanced in primary t(8;21) positive patient cells and myeloid cell lines?

Is c-jun a direct target of AML1-ETO and what is the role of the JNK signaling pathway, which can phosphorylate Ser63 in c-Jun, in myeloid cells?

Is the JNK signaling pathway of importance for the functional and biological properties of t(8;21) positive AML?

- AML1-ETO has no known kinase activity. It is currently unknown how AML1-ETO enhances the phosphorylation of a protein.

What might be the underlying molecular mechanism of AML1-ETO influencing a signaling pathway?

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## 3. MATERIALS

### 3.1 Cell lines

#### U937

Human histiocytic lymphoma. Source: ACC5, DSMZ, Braunschweig, Germany

#### U937 WITH ZINC-INDUCIBLE EXPRESSION OF AML1-ETO (U937Z/A-E)

Source: Pier Giuseppe Pelicci, Istituto Europeo di Oncologia, Milano

#### U937 WITH TET-REGULATED EXPRESSION OF AML1-ETO (U937T/A-E)

Source: Dong-Er Zhang, The Scripps Research Institute, La Jolla<sup>88</sup>

#### U937 TET (U937T)

Control cell line containing the tetracycline-responsive transcriptional repressor tTA. Source: Dong-Er Zhang, The Scripps Research Institute, La Jolla<sup>88</sup>

#### KASUMI-1

Human acute myeloid leukemia. Source: ACC220, DSMZ, Braunschweig, Germany

#### 293T

Human embryonal kidney. Source: ACC305, DSMZ, Braunschweig, Germany

### 3.2 Patient material

Patient samples were referred to the Laboratory for Leukemia Diagnostics, Department of Medicine III, Klinikum Großhadern, Munich, for routine cytogenetic and cytomorphic analyses. All samples were from the time of diagnosis. AML patient samples analyzed by microarray analysis had only a single translocation, the group of t(11q23/MLL) positive patient samples contained one sample with a t(11;19), one sample with a t(6;11), one with a t(11;22) and seven samples with a t(9;11).



In the t(8;21) positive patient samples analyzed by real-time PCR, thirteen patients had an AML FAB subtype M2 and in three cases the AML FAB subtype had not been analyzed (patient numbers 8, 10 and 15, Figure 2). Three patients had a t(8;21) as sole cytogenetic abnormality (patient numbers 7, 11 and 16, Figure 2), thirteen had loss of a sex chromosome, two had trisomy 8 (patient numbers 2 and 10, Figure 2) and one a t(15;20) (patient number 10, Figure 2). The AML1-ETO transcript was observed in all cases (patient numbers 1 to 16, Figure 2).

### **3.3 Plasmids, reporter constructs, primers, oligonucleotides**

#### PLASMIDS

##### **pcDNA3.1**

Eukaryotic expression vector. Source: Invitrogen, Groningen, The Netherlands

##### **pCMV5**

Eukaryotic expression vector, 4.7Kb, with ampicillin resistance gene and strong CMV promoter. Source: Amersham Pharmacia, Freiburg, Germany

##### **pRL-null**

Control reporter vector for the Dual-luciferase reporter assay system containing the cDNA encoding *Renilla* luciferase without eukaryotic promoter and/or enhancer elements. Source: Promega, Madison, WI, USA

##### **pGdBBX**

Full-length G6PD cDNA in the expression vector pUC12.

Source: Andreas Hochhaus, III. Medizinische Universitätsklinik, Mannheim, Germany<sup>98</sup>

##### **Human pCMV5 AML1-ETO**

Source: Scott Hiebert, St. Jude Children's Hospital, Memphis, USA

**Human pCMV5 ETO**

(S. Hiebert)

**Human pCMV5 CBF $\beta$** 

(S. Hiebert)

**Human pCMV5 AML1**

(S. Hiebert)

**Murine pSV-SPORT1 c-jun**

Source: Trang Hoang, Clinical Research Institute of Montreal, QC, Canada

**Human pCMV5 JIP-1**

Source: Roger Davis, Howard Hughes Medical Institute, Massachusetts, USA

**Murine pSP65 c-jun**

c-jun for *in vitro* translation using SP6 polymerase. Source: Elisabetta Mueller and Bruce Spiegelman, Dana Farber Cancer Institute, Boston, MA<sup>99</sup>

## REPORTER CONSTRUCTS

**Human c-jun promoter [bp -1780 to bp +731] in pGL3**

Source: Wayne Vedeckis, State University Medical Center, New Orleans, USA

**Human c-jun promoter [bp -952 to bp +731] in pGL3 (W. Vedeckis)****Human c-jun promoter [bp -719 to bp +731] in pGL3 (W. Vedeckis)****Human c-jun promoter [bp -345 to bp +731] in pGL3 (W. Vedeckis)****Human c-jun promoter [bp -180 to bp +731] in pGL3 (W. Vedeckis)****Human c-jun promoter [bp -63 to bp +731] in pGL3 (W. Vedeckis)**

**Human c-jun promoter [bp -79 to bp +170] in pGL2**

Source: Xiao-Fan Wang, Duke University Medical Center, Durham, North Carolina, USA

**Human c-jun promoter [bp -79 to bp +170] in pGL2 with the proximal AP-1 site: 5'-tg aca tca-3' mutated to 5'-at cca cca-3' (X.-F. Wang)****Minimal c-fos promoter element containing 3x NF- $\kappa$ B binding sites in pGL2**

Source: Marius Ueffing, Institute of Human Genetics, GSF, Munich, Germany

**Minimal c-fos promoter element containing 3x mutated NF- $\kappa$ B binding sites in pGL2 (Marius Ueffing)****Human M-CSF receptor promoter [bp -416 to bp +71] in pXP2**

Source: D.E. Zhang, Scripps Research Institute, La Jolla, CA, USA<sup>92</sup>

**pGL2, pGL3**

Luciferase reporter gene vectors. Source: Promega, Mannheim, Germany

**pXP2**

Luciferase reporter gene vector. Source: Stephen Nordeen, Department of Pathology, University of Colorado, Denver, USA

**PRIMERS**

**c-jun Sense:** 5'-gca tga gga acc gca tgc ctg cct cca agt-3'<sup>100</sup>

**c-jun Antisense:** 5'-gcg acc aag tcc ttc cca ctc gtg cac act-3'<sup>100</sup>

**G6PD Sense:** 5'-ccg gat cga cca cta cct ggg caa g-3'<sup>98</sup>

**G6PD Antisense:** 5'-ggt ccc cac gta ctg gcc cag gac ca-3'<sup>98</sup>

**AML1-ETO Sense:** 5'-atg acc tca ggt ttg tgc gtc g-3'

**AML1-ETO Antisense:** 5'-tga act ggt tct tgg agc ctc ct-3'

**G-CSF receptor Sense:** 5'-cct gga gct gag aac tac cg-3'<sup>101</sup>

**G-CSF receptor Antisense:** 5'-tcc cgg ctg agt tat agg-3'<sup>101</sup>

**G-CSF Sense:** 5'-gct tga gcc aac tcc ata gc-3'

**G-CSF Antisense:** 5'-aaa tgc agg gaa gga cac ag-3'

**pcjun -63/+170 Sense:** : 5'-tgg gct att ttt agg ggt tga ctg g-3'

**pcjun -63/+170 Antisense:** 5'-agc cac agg cgc tag ctc tgg g-3'

#### OLIGONUCLEOTIDES FOR EMSA

##### **Human c-jun promoter [bp -88 to bp -28]**

5'-tgg gaa ggc ctt ggg gtg aca tca tgg gct att ttt agg ggt tga ctg gta gca gat aag-3'

##### **Human c-jun promoter [bp -88 to bp -28] with mutated proximal AP-1 site**

5'-tgg gaa ggc ctt ggg gat cca cca tgg gct att ttt agg ggt tga ctg gta gca gat aag-3'

##### **AML1 consensus site**<sup>62</sup>

5'-aat tcg agt att gtg gtt aat acg-3'

## 3.4 Antibodies, peptides

#### ANTIBODIES

##### **anti c-Jun/AP-1 (N)**

Polyclonal rabbit antibody against the amino terminal domain of c-Jun. Dilution for Western blot: 1:1,000. Used also in EMSA (200µg/0.1ml). Source: Santa Cruz Biotechnology, Santa Cruz, CA

##### **anti ETO (C-20)**

Polyclonal goat antibody against the carboxy terminus of ETO. Dilution for Western blot: 1:1,000. Used also in EMSA (200µg/0.1ml). Source: Santa Cruz

##### **anti b-tubulin (H-235)**

Polyclonal rabbit antibody against amino acids 210-444 at the carboxy terminus of β-tubulin. Dilution: 1:500. Source: Santa Cruz

**anti JNK1 (C-17)**

Polyclonal rabbit antibody against the carboxy terminus of JNK1. Dilution: 1:1,000.  
Source: Santa Cruz

**anti AML1 (N-20)**

Polyclonal goat antibody against the amino terminus of AML1. Used in EMSA (200µg/0.1ml). Source: Santa Cruz

**anti c-fos (4)**

Polyclonal rabbit antibody against the amino terminus of c-fos. No cross-reactivity to Fos B, Fra-1 or Fra-2. Used in EMSA (200µg/0.1ml). Source: Santa Cruz

**anti ATF-2 (F2BR-1)**

Mouse monoclonal antibody against amino acids 350-505 within the DNA binding and dimerization domain of ATF-2. Used in EMSA (200µg/0.1ml).  
Source: Santa Cruz

**anti GST (Z-5)**

Rabbit polyclonal antibody against GST fusion proteins. Dilution: 1:1,000.  
Source: Santa Cruz

**normal IgG rabbit**

Isotype control for rabbit antibodies. Source: Santa Cruz

**normal IgG goat**

Isotype control for goat antibodies. Source: Santa Cruz

**anti phosphorylated Thr183/Tyr185 in SAPK/JNK**

Polyclonal rabbit antibody. Detects SAPK/JNK only when activated by phosphorylation at Thr183/Tyr185. Dilution: 1:1,000. Source: New England Biolabs, Schwalbach, Germany

**anti phosphorylated Thr71 in ATF-2**

Polyclonal rabbit antibody. Recognizes ATF-2 only when phosphorylated at Thr71.  
Dilution: 1:1,000. Source: New England Biolabs

**anti phosphorylated Ser63 in c-Jun**

Polyclonal rabbit antibody, detects phosphorylated Ser63 in c-Jun, but does not cross-react with the corresponding phosphorylated forms of JunD or JunB.  
Dilution: 1:1,000. Source: New England Biolabs

**anti phosphorylated Ser73 in c-Jun**

Polyclonal rabbit antibody, detects phosphorylated Ser73 in c-Jun and Ser100 in JunD, but does not cross-react with up to 0.5µg of nonphosphorylated c-Jun or JunD. Dilution: 1:1,000. Source: New England Biolabs

**donkey anti rabbit IgG (NA934)**

HRP-conjugated secondary antibody. Dilution: 1:2,000. Source: Amersham Pharmacia, Freiburg, Germany

**rabbit anti goat (P0449)**

HRP-conjugated secondary antibody. Dilution: 1:2,000. Source: DAKO, Denmark

## PEPTIDES

**JNK inhibitor I (L)-form**

A cell permeable, biologically active peptide that diminishes JNK signaling pathway by blocking the activation of transcription factor c-Jun. 20 amino acids of the islet-brain-1 protein, an isoform of JIP-1, inhibiting JNK induced phosphorylation of c-Jun by interacting in the JNK binding domain (delta domain) of c-Jun, are fused to 10 amino acids of a HIV-TAT carrier, which rapidly translocates into the cells. Source: Calbiochem, Darmstadt, Germany<sup>102</sup>

**HIV-TAT 48-57, Negative control**

A highly cell-permeable 10-amino acid carrier peptide derived from HIV-TAT 48-57 sequence that is modified with two proline residues. Source: Calbiochem, Darmstadt, Germany<sup>102</sup>

**3.5 Laboratory materials****CHEMICALS**

Acrylamide/Bisacrylamide 40% (Serva, Heidelberg, Germany)

Agarose (Serva, Heidelberg, Germany)

Ammonium peroxodisulfate (APS) (Bio-rad, Munich, Germany)

[ $\gamma$ -<sup>32</sup>P]ATP (3000Ci/mmol at 10mCi/ml) (Amersham Pharmacia, Freiburg, Germany)

Bio-rad Protein estimation kit (Bio-rad, Munich, Germany)

Detection system ECL<sup>®</sup> (Amersham Pharmacia, Freiburg, Germany)

DNA molecular weight markers (Roche Diagnostics, Mannheim, Germany)

dNTPs (Promega, Mannheim, Germany)

Dual-luciferase reporter assay system (Promega, Mannheim, Germany)

Dulbeccos modified Eagle medium (DMEM) (PAN, Aidenbach, Germany)

Effectene<sup>®</sup> transfection kit (Qiagen, Hilden, Germany)

Ethidium bromide (Life Technologies, Karlsruhe, Germany)

Fast Start DNA SYBR Green I kit (Roche Diagnostics, Mannheim, Germany)

Genzyme TACS<sup>™</sup> AnnexinV kit (R&D systems, Wiesbaden, Germany)

Granocyte<sup>®</sup> 34: Human recombinant G-CSF (Chugai, Frankfurt, Germany)

L-Glutamine (Life Technologies, Karlsruhe, Germany)

Liquid nitrogen (Linde, Munich, Germany)

Maxiprep<sup>®</sup> DNA isolation kit (Qiagen, Hilden, Germany)

Onmascript<sup>®</sup> RT cDNA synthesis kit (Qiagen, Hilden, Germany)

PBS (PAN, Aidenbach, Germany)

PCR primers, oligo dT (Metabion, Martinsried, Germany)

Penicillin-Streptomycin (Life Technologies, Karlsruhe, Germany)

poly(dI-dC) (Amersham Pharmacia, Freiburg, Germany)

Protein markers (Amersham Pharmacia, Freiburg, Germany)

Restriction enzymes (New England Biolabs, Schwalbach, Germany)

RNasin<sup>®</sup> RNase inhibitor (Promega, Mannheim, Germany)

RNeasy<sup>®</sup> RNA isolation kit (Qiagen, Hilden, Germany)

RPMI 1640 medium (PAN, Aidenbach, Germany)

SAPK/JNK assay kit (Cell signaling technology, Beverly, USA)

SDS (Bio-rad, Munich, Germany)

T4 polynucleotide kinase (New England Biolabs, Schwalbach, Germany)

Taq polymerase (Qiagen, Hilden, Germany)

TnT<sup>®</sup> reticulocyte lysate system (Promega, Mannheim, Germany)

TOPO TA PCR2.1 (Invitrogen, Groningen, The Netherlands)

Tran <sup>35</sup>S-label<sup>TM</sup> (>1000Ci/mmol at 10mCi/ml) (ICN Biomedicals, Eschwege, Germany)

Tris (Bio-rad, Munich, Germany)

Trypan blue 0.5% (Serva, Heidelberg, Germany)

Trypsin (PAN, Aidenbach, Germany)

All the other chemicals were obtained from Sigma (St. Louis, USA).

#### LABORATORY EQUIPMENT, CONSUMABLES

Incubator for bacteria (Heraus, Hanau, Germany)

Shaker for bacteria (New Brunswick Scientific, Nürtingen, Germany)

Blotting paper (Schleicher& Schüll, Dassel, Germany)

ECL<sup>®</sup> films (Amersham Pharmacia, Freiburg, Germany)

Eppendorf<sup>®</sup> tabletop centrifuge 5415D (Eppendorf, Hamburg, Germany)



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Developing machine (Agfa-gevaert, Leverkusen, Germany)

Gel electrophoresis systems (Bio-rad, Munich, Germany; OWL, Portsmouth, USA)

Heating block (Techne, Cambridge, UK)

Immobilon<sup>TM</sup>-P transfer membranes (Millipore, Bedford, Massachusetts, USA)

Parafilm M<sup>®</sup> (American National Can, Greenwich, USA)

Thermal cycler (Perkin Elmer, Norwalk, USA)

Light Cycler<sup>TM</sup> real-time PCR machine (Roche Diagnostics, Mannheim, Germany)

Luminometer Turner Designs TD-20/20 (Promega, Madison, WI, USA)

pH-meter (WTW, Weilheim, Germany)

Photometer (Amersham Pharmacia, Freiburg, Germany)

Pipettes (Gilson, Langenfeld, Germany)

Pipette tips (Star Labs, K&K Laborbedarf, Munich, Germany)

QuickSpin<sup>®</sup> columns (Roche Diagnostics, Mannheim, Germany)

Freezing vials (Nunc, Roskilde, Danmark)

Liquid nitrogen tank (Cryoson, Schöllkrippen, Germany)

Cell culture incubator (WTB, Tuttlingen, Germany)

Microscope (Zeiss, Oberkochen, Germany)

Pipettes, Tissue culture flasks, Centrifuge vials (Sarstedt, Nümbrecht, Germany)

Sterile benches (BDK, Sonnenbühl, Germany)

Sterile filters (Gelman Sciences, Ann Arbor USA)

Neubauer<sup>®</sup> counting chamber (Brand, Wertheim, Germany)

### 3.6 Buffer, media, solutions

#### ELECTROPHORESIS BUFFER (SDS-PAGE)

25mM Tris, pH 8.3

250mM Glycine

0.1% (w/v) SDS

#### SDS/ACRYLAMIDE GEL FOR WESTERN BLOT

**Running gel (10%)**

40% (v/v) Acrylamide

1.5M Tris, pH 8.8

10% (w/v) SDS

10% (w/v) APS

0.0005% (v/v) TEMED

**Stacking gel (10%)**

40% (v/v) Acrylamide

0.5M Tris, pH 6.8

10% (w/v) SDS

10% (w/v) APS

0.0005% (v/v) TEMED

#### TAE (TRIS/ACETATE/EDTA) ELECTROPHORESIS BUFFER

40mM Tris acetate

2mM Na<sub>2</sub>EDTA·2H<sub>2</sub>O

#### TBE (TRIS/BORATE/EDTA) ELECTROPHORESIS BUFFER

890mM Tris

890mM Boric acid

10mM EDTA, pH 8.0

#### SDS-PAGE GEL LOADING DYE (2X)

125mM Tris·Cl, pH 6.8

4% (w/v) SDS

10% (v/v) β-Mercapto-ethanol

30% (v/v) Glycerol

0.004% (w/v) Bromophenol blue

WESTERN BLOT STRIPPING SOLUTION

0.1M  $\beta$ -Mercapto-ethanol  
2% (w/v) SDS  
1M Tris, pH 6.8

TE BUFFER

10mM Tris·Cl, pH 8.0  
1mM EDTA

ELECTROPHORESIS TANK BUFFER (WESTERN BLOT)

125mM Tris  
960mM Glycine  
0.5% (w/v) SDS

TRANSFER BUFFER, pH 8.5

25mM Tris  
0.2M Glycine  
20% (v/v) Methanol

TRIS-BUFFERED SALINE (TBS)

100mM Tris·Cl, pH 7.5  
150mM NaCl

TBST (TBS PLUS TWEEN20)

100mM Tris·Cl, pH 7.5  
150mM NaCl  
0.05% (v/v) Tween20

BLOCKING BUFFER

TBS  
0.1% (v/v) Tween20  
5% (w/v) nonfat dry milk

PRIMARY ANTIBODY DILUTION BUFFER

TBS

0.05% (v/v) Tween20

2.5% (w/v) nonfat dry milk

PRIMARY ANTIBODY DILUTION BUFFER

(FOR PHOSPHORYLATED PROTEINS)

TBS

0.05% (v/v) Tween20

5% (w/v) BSA

WHOLE CELL LYSIS BUFFER (RIPA LYSIS BUFFER)

1% (v/v) NP40

0.5% (w/v) Sodium deoxycholate

0.1% (w/v) SDS

0.15M NaCl

5mM EDTA

50mM Tris, pH 8.0

10mM Sodium pyrophosphate

50mM Sodium fluoride

0.2mM Sodium orthovanadate

1mM PMSF

10mg/ml Pepstatin A, Leupeptin, Aprotinin, Antipain, Chymostatin

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**NUCLEAR EXTRACT BUFFERS****Buffer A:**

20mM HEPES, pH 7.9  
25% (v/v) Glycerol  
420mM NaCl  
1.5mM MgCl<sub>2</sub>  
0.2mM EDTA  
0.5mM DTT  
0.2mM PMSF

**Buffer C:**

10mM HEPES, pH 7.9  
1.5mM MgCl<sub>2</sub>  
10mM KCl  
0.5mM DTT  
0.2mM PMSF

**EMSA BINDING BUFFER**

4mM HEPES, pH 7.9  
40mM KCl  
10mM MgCl<sub>2</sub>  
1mM Dithiothreitol (DTT)  
0.2mM Phenylmethylsulfonyl fluoride (PMSF)  
4% (v/v) Glycerol

**10X ANNEALING BUFFER FOR EMSA**

20mM Tris, pH 7.5  
10mM MgCl<sub>2</sub>  
50mM NaCl  
1mM DTT

**TRIS-GLYCINE ELECTROPHORESIS BUFFER FOR EMSA, pH 8.3**

0.25M Tris  
1.9M Glycine  
10mM EDTA

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#### 5.2% POLYACRYLAMIDE GEL FOR EMSA

40% (v/v) Bisacrylamide 1:19  
890mM Tris  
890mM Boric acid  
10mM EDTA, pH 8.0  
10% (w/v) APS  
0.0005% TEMED

#### 5X BINDING BUFFER FOR EMSA

4mM HEPES, pH 7.9  
40mM KCl  
10mM MgCl<sub>2</sub>  
1mM DTT  
0.2mM PMSF  
4% (v/v) Glycerol

#### GROWTH MEDIUM FOR BACTERIA

LB (*luria bertani*) medium with respective antibiotics

#### MAMMALIAN CELL CULTURE MEDIA

##### **293T cell line**

DMEM  
10% (v/v) FBS  
1% (v/v) L-Glutamine  
1% (v/v) Penicillin-Streptomycin

##### **U937, U937/Zn<sup>2+</sup>-inducible AML1-ETO and Kasumi-1 cell lines**

RPMI  
10% (v/v) FBS  
1% (v/v) L-Glutamine  
1% (v/v) Penicillin-Streptomycin

**U937/TET cell line**

RPMI

10% (v/v) FBS

1% (v/v) L-Glutamine

1 $\mu$ g/ml Tetracycline

0.5 $\mu$ g/ml Puromycin

**U937/TET/AML1-ETO cell line**

RPMI

10% (v/v) FBS

1% (v/v) L-Glutamine

1 $\mu$ g/ml Tetracycline

0.5 $\mu$ g/ml Puromycin

1mg/ml G418

Other buffers were provided by kits.

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## 4. METHODS

### 4.1 Cell culture methods

#### INDUCIBLE CELL LINES

##### **U937/Zn<sup>2+</sup>-inducible AML1-ETO (U937Z/A-E)**

AML1-ETO cDNA is under the control of the human metallothionein promoter in the expression vector pPC18. Adding 100 $\mu$ M Zn<sup>2+</sup> to the cell culture medium induced the expression of AML1-ETO.

##### **U937/TET-regulated AML1-ETO (U937T/A-E)**

AML1-ETO cDNA is under the control of the tetracycline-responsive transcriptional repressor tTA. For the induction of tetracycline-controlled AML1-ETO, cells were washed three times in 50ml of PBS and seeded at 1x10<sup>5</sup> cells/ml in the maintenance medium in the absence of tetracycline.

#### PRIMING WITH G-CSF

U937T/A-E cells were grown for 48 hours with or without tetracycline and subsequently treated with 10 $\mu$ M or 100 $\mu$ M of human recombinant G-CSF for 15 or 30 minutes. RIPA lysates were prepared and analyzed by Western blot for the amount of phosphorylated Thr183/Tyr185 in JNK.

#### JNK INHIBITOR PEPTIDE

U937T/A-E and U937T cells were grown for 72 hours in the presence or absence of tetracycline. 1 $\mu$ M of JNK specific inhibitor peptide or HIV-TAT 48-57 negative peptide control was added to the cells at the time of induction.



#### SUPERNATANT EXPERIMENTS

Supernatants of  $1 \times 10^7$  U937T/A-E and U937T cells were harvested 48 hours after removal of tetracycline.  $1 \times 10^7$  wildtype U937 cells were centrifuged, the supernatant removed and the cell pellet resuspended with the supernatants of U937T/A-E or U937T cells. Wildtype U937 cells were incubated for 15 or 30 minutes with the supernatants and immediately kept on ice. Whole cells were lysed using RIPA buffer (see buffers) and the lysates were kept at  $-80^\circ\text{C}$ . Lysates were analyzed by Western blot for expression of phosphorylated Ser63 in c-Jun and phosphorylated Thr183/Tyr185 in JNK.

#### 4.2 Transient transfections using Effectene<sup>®</sup> reagent

Transient transfection using the Effectene<sup>®</sup> transfection kit allows transfection in the presence of FBS, which was preferred in this study because of the serum induced fluctuations in c-Jun expression.

##### FOR ADHERENT CELLS

293T cells were plated 24 hours prior to transfection at about  $5 \times 10^5$  cells/ml in  $75\text{cm}^2$  flasks to achieve a 50-60% confluence on the day of transfection. In a 1.5ml Eppendorf tube,  $2\mu\text{g}$  of DNA was diluted in  $300\mu\text{l}$  of EC buffer (DNA condensation buffer, Effectene<sup>®</sup> transfection kit) and  $16\mu\text{l}$  of Enhancer (Effectene<sup>®</sup> transfection kit). The Enhancer forms complexes with the transfected DNA. Complexes were vortexed for 1 second and incubated for 2 to 5 minutes at room temperature.  $20\mu\text{l}$  of Effectene<sup>®</sup> transfection reagent (Effectene<sup>®</sup> transfection kit) was added to the DNA enhancer mixture. The complex was vortexed for 10 seconds and incubated at room temperature for 5 to 10 minutes to allow transfection complex formation. During this period, the growth medium was gently aspirated from the cells and 12ml of fresh DMEM with FBS and antibiotics was added. The transfection complexes were pipetted drop-wise onto the cells with 1ml of DMEM containing FBS.

The dish was gently swirled to ensure uniform distribution of the transfection complexes and cells were incubated for 48 hours at  $37^\circ\text{C}$  at 5%  $\text{CO}_2$ .

#### FOR SUSPENSION CELLS

24 hours prior to transfection, U937 cells were split to  $3 \times 10^5$  cells/ml. On the day of transfection,  $1 \times 10^6$  cells were plated in 1.6ml of medium with FBS and antibiotics into 6 well plates. 1 $\mu$ g of DNA was diluted in 100 $\mu$ l of EC buffer (Effectene<sup>®</sup> transfection kit). 6 $\mu$ l Enhancer and 20 $\mu$ l of Effectene<sup>®</sup> transfection reagent (Effectene<sup>®</sup> transfection kit) were added, the solution was applied to the cell suspension with 600 $\mu$ l of RPMI medium with FBS and antibiotics and incubated for 30 hours.

U937T or U937T/A-E cells were kept in the maintenance medium with or without tetracycline for 12 hours prior to transfection and harvested 24 hours after transfection.

#### 4.3 PCR cloning

For generation of a human c-jun promoter construct ranging from bp -63 to bp +170 in pGL2, the fragment was amplified by PCR using pcjun -63/+170 Sense and Antisense primers (see primers). The c-jun promoter construct ranging from bp -79 to bp +170 served as a template. 1 $\mu$ l of the PCR product was immediately cloned into the plasmid vector pCR2.1 according to TOPO TA cloning strategy. Subsequently, the fragment was released by *Kpn/XhoI* digest and subcloned into the *Kpn/XhoI* site of pGL2 luciferase vector.

## 4.4 Reverse transcriptase PCR

### ISOLATION OF TOTAL RNA ACCORDING TO THE RNEASY<sup>®</sup> METHOD

$3 \times 10^6$  suspension cells (cell lines or peripheral blood monocytes of patient samples) were washed twice with PBS and transferred with 1ml of PBS into 1.5ml Eppendorf tubes. To disrupt and lyse the cells, cells were spun at 300g for 5 minutes and resuspended in 350 $\mu$ l of RLT buffer (RNeasy<sup>®</sup> RNA isolation kit). For homogenization, the lysate was transferred to a QIAshredder<sup>®</sup> column (RNeasy<sup>®</sup> RNA isolation kit) and centrifuged for 2 minutes at maximum speed in an Eppendorf tabletop centrifuge. 350 $\mu$ l of 70% ethanol were added to adjust the binding conditions and the sample was added to an RNeasy<sup>®</sup> mini spin column (RNeasy<sup>®</sup> RNA isolation kit), in which the RNA could absorb to the membrane. Contaminants were removed by wash spins at 10,000 rpm for 15 seconds with 700 $\mu$ l of buffer RW1 (RNeasy<sup>®</sup> RNA isolation kit) and with 500 $\mu$ l of RPE buffer (RNeasy<sup>®</sup> RNA isolation kit). To wash out all of the ethanol, 500 $\mu$ l RPE buffer was added and another 2 minute-spin at maximum speed was performed. For RNA elution, the column was placed in a 1.5ml collection tube and 40 $\mu$ l of RNase-free water was directly pipetted onto the column and centrifuged for 1 minute at 10,000 rpm. Total RNA was stored at -80°C.

### FIRST-STRAND cDNA SYNTHESIS

Reverse transcriptases (RT) with RNA dependent DNA polymerase activity synthesize complementary DNA (cDNA) from an RNA template for subsequent PCR or cloning experiments. 1 $\mu$ g of RNA was transcribed in 20 $\mu$ l of reverse transcriptase reaction containing 2 $\mu$ l of 5mM dNTPs, 2 $\mu$ l of 10x RT buffer (Omniscript<sup>®</sup> RT kit), 2 $\mu$ l of 10 $\mu$ M 17mer oligo dT primer, 1 $\mu$ l of 10U/ $\mu$ l RNasin<sup>®</sup> RNase inhibitor, 1 $\mu$ l of 4U/ $\mu$ l Omniscript<sup>®</sup> reverse transcriptase (Omniscript<sup>®</sup> RT kit) and RNase-free water up to 20 $\mu$ l. The cDNA synthesis mix was incubated for 90 minutes at 37°C and the cDNA was stored at -20°C.

#### SEMI-QUANTITATIVE RT-PCR

We performed semi-quantitative hot-start PCR using a thermal cycler in order to optimize PCR conditions prior to performing quantitative real-time PCR. PCR was performed in 20 $\mu$ l containing 2 $\mu$ l cDNA template, 200 $\mu$ M of each dNTP, 1.5mM MgCl<sub>2</sub>, 0.7 $\mu$ M of sense and antisense primer and 2.5U Taq polymerase. PCR products were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining.

#### QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR using the LightCycler<sup>TM</sup> (LC) real-time PCR system enables real-time monitoring of PCR product formation. PCR cycles, in which the PCR product increases logarithmically, can be identified and the initial concentration of the target DNA determined. We used the Fast Start DNA SYBR Green I kit. SYBR Green I dye is a fluorescence dye, which binds to double-stranded DNA. A fluorescence signal is being recorded at the end of each elongation phase and the increasing amounts of PCR product can be monitored from cycle to cycle.

We quantified the expression of the transcription factors c-jun and AML1-ETO in U937 cell lines with Zn<sup>2+</sup>-inducible expression of AML1-ETO and in t(8;21) positive patient samples relative to the expression of the housekeeping gene G6PD. We performed relative quantification of the target genes by forming ratios between the target genes and the housekeeping gene G6PD (target gene/G6PD). The initial concentrations of the target genes and the housekeeping gene G6PD were calculated using a standard curve. For this purpose, we serially diluted a G6PD plasmid: pGdBBX to 10,000fg, 1,000fg or 100fg. Upregulation of c-jun upon AML1-ETO induction or the upregulation of AML1-ETO upon addition of Zn<sup>2+</sup> were shown as fold upregulation compared to time point zero, which was set to 1. For the patient samples, values of target gene/G6PD ratios were directly compared to each other.

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PCR was performed using 2 $\mu$ l of 10x mastermix solution (provided, contains Fast-Start Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and 10mM MgCl<sub>2</sub>), 2 $\mu$ l cDNA (from cell lines or patient samples), 4mM MgCl<sub>2</sub>, 7.5 $\mu$ M of each primer and RNase-free water to a final volume of 20 $\mu$ l. After 10 minutes initial denaturation at 95°C to activate the polymerase, amplification occurred in a three-step cycle procedure with denaturation at 95°C for 0 seconds, annealing at 64°C for 10 seconds and extension at 72°C for 25 seconds. This three-step cycle was repeated 35 times. Fluorescence of SYBR Green I was measured after each extension step at 530nm. The final PCR cycle was followed by a melting curve analysis to confirm PCR product identity and differentiate it from non-specific products (for example primer-dimer products). For the melting curve analysis, the products were denatured at 95°C, annealed at 65°C and then slowly heated up to 95°C with fluorescence measurement at 0.2°C increments. Amplification products from the LightCycler were checked by electrophoresis on 1% ethidium bromide stained agarose gels. The estimated size of the amplified fragments matched the calculated size: for c-jun: 409bp, G6PD: 343bp, G-CSF receptor: 276bp, G-CSF: 591bp and AML1-ETO: 250bp.

#### 4.5 Reporter assay for *Firefly* and *Renilla* luciferase

We studied the effects of leukemic fusion genes or transcription factors on gene expression by transiently transfecting cells with plasmids containing the relevant promoters 5' of a *Firefly* luciferase reporter gene. All samples were transfected with a *Renilla* luciferase “control” reporter plasmid. Normalizing the activity of the experimental reporter to the activity of the internal control reporter minimized experimental variability caused by differences in cell viability or transfection efficiency. The activities of *Firefly* luciferase of the experimental reporter and *Renilla* luciferase of the pRL-null control plasmid were measured sequentially from a single sample using the Dual-luciferase reporter assay system.

Transfected cells were washed twice with PBS and lysed in 100µl of Passive lysis buffer (Dual-luciferase reporter assay system) by agitation for 30 minutes. Passive Lysis buffer was provided as a 5x concentrate, diluted in ddH<sub>2</sub>O and stored at 4°C up to one month.

The Luciferase assay reagent II (LARII) (Dual-luciferase reporter assay system) was resuspended in 10ml of Luciferase assay buffer and was storable for 1 month at -20°C or for 1 year at -70°C. The 50X Stop&Glo stock solution (Dual-luciferase reporter assay system), used to quench *Firefly* luciferase luminescence, was prepared by transferring 200µl of Stop&Glo substrate solvent (Dual-luciferase reporter assay system) to the lyophilized Stop&Glo substrate (Dual-luciferase reporter assay system). This solution has to be kept on ice and was storable at -70°C for 1 month. Immediately before use, 1x Stop&Glo working solution was prepared with Stop&Glo buffer (Dual-luciferase reporter assay system) and kept on ice.

20µl of cell lysate was diluted in 100µl of LARII and the *Firefly* luciferase luminescence of the transfected reporter plasmid measured using the TD20/20 luminometer. After addition of 100µl Stop&Glo working solution, which quenched the *Firefly* luciferase, the remaining *Renilla* luciferase of the internal control plasmid pRL-null was measured. A ratio between *Firefly* and *Renilla* luciferase was calculated automatically.

## 4.6 Cell lysates, *in vitro* translation

### RIPA LYSATES

Adherent cells were scraped in 10ml of PBS. Subsequently, approximately  $1 \times 10^7$  cells were washed twice in PBS by centrifuging the cells at 1,000 rpm for 10 minutes. Suspension cells were washed directly. Washed cells were resuspended in 1ml of PBS, transferred to 1.5ml Eppendorf tubes and spun for 10 seconds at maximum speed. The pellet was resuspended in 50µl of RIPA lysis buffer with proteinase and phosphatase inhibitors (see buffers) and incubated on ice for 30 minutes.

During incubation time, cell lysates were vortexed every 10 minutes. After incubation, lysates were spun at 4°C for 30 minutes at maximum speed. The supernatant was collected and stored immediately at -80°C.

#### NUCLEAR EXTRACTS

Adherent cells were scraped in 10ml of ice-cold PBS and approximately  $1 \times 10^7$  cells were washed twice in PBS at 1,000 rpm for 10 minutes. Suspension cells were washed directly. Cell pellets were resuspended in 1ml of PBS, transferred to 1.5ml Eppendorf tubes and spun at 6,600 rpm for 20 seconds. Pellets were resuspended in 400µl cold Buffer A (see buffers), were swollen on ice for 15 minutes and vortexed for 10 seconds. The nuclei were pelleted in a tabletop centrifuge for 10 seconds at maximum speed. Subsequently, the nuclei pellets were resuspended in 40µl of Buffer C (see buffers) by pipetting up and down. This was followed by a 20-minute incubation period on ice, during which the tubes were flicked occasionally. The cell debris was pelleted at maximum speed for 3 minutes at 4°C and the supernatants were saved and stored at -80°C.

#### IN VITRO TRANSLATION

AML1-ETO and c-jun were *in vitro* transcribed and translated using the TnT<sup>®</sup> reticulocyte lysate system with SP6 polymerase and were labeled at amino acid methionine with Tran <sup>35</sup>S-label<sup>TM</sup> (>1000Ci/mmol at 10mCi/ml). For a 50µl reaction we used:

TnT <sup>®</sup> rabbit reticulocyte lysate (provided)	25µl
TnT <sup>®</sup> reaction buffer (provided)	2µl
SP6 polymerase	1µl
amino acid mixture minus methionine (provided)	1µl
Tran <sup>35</sup> S-label <sup>TM</sup> (>1000Ci/mmol at 10mCi/ml)	4µl
RNasin <sup>®</sup> RNase inhibitor (40U/µl)	1µl
DNA template (1µg)	variable
RNase-free water	up to 50µl

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Reagents were mixed and incubated at 30°C for 90 minutes.

## 4.7 Western blot analysis

### PROTEIN MEASUREMENT

For measuring total protein concentration, we performed a colorimetric assay based on the Bradford-dye binding procedure<sup>103</sup> according to the protocol of the Bio-rad protein estimation kit. The assay is based on the color change of Coomassie blue in response to various concentrations of protein. We mixed 20µl of 1:20 diluted protein lysate with 1ml of 1:5 diluted reagent dye, incubated the mixture for 15 minutes and determined the color change at 595nm using a spectrophotometer. We included BSA protein standards of known concentrations for generating a standard curve with which we could calculate the protein concentration in µg/µl.

### SDS-PAGE AND WESTERN BLOT

Lysates containing 100µg of proteins were diluted in 2x SDS-PAGE gel loading dye (see buffers) and boiled at 95°C for 5 minutes. Proteins were electrophoretically separated according to their molecular weight through a 10% SDS/Acrylamide gel (see buffers) at 125 volts. 10µl of molecular weight standard was included in each gel. The separated proteins were wet-transferred to Immobilon<sup>TM</sup>-P transfer membranes, which had been pre-soaked in methanol, rinsed with ddH<sub>2</sub>O and kept in transfer buffer (see buffers) for 15 minutes. The transfer cassette was prepared in the following sequence: sponge > blotting paper > gel > transfer membrane > blotting paper > sponge. The transfer chamber was filled with transfer buffer and the proteins were transferred at 100 volts for 90 minutes at 4°C. After the transfer, the membrane was blocked for 2 hours in a blocking buffer (see buffers) to prevent unspecific binding of the antibodies to the membrane. The blocked membrane was washed three times with TBST (see buffers) to get rid of excess blocking solution and the primary antibody was added at a dilution of 1:1,000 and incubated overnight at 4°C.



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Antibodies recognizing phosphorylated proteins were diluted in TBST with 1% (w/v) BSA, antibodies against unphosphorylated proteins in TBST with 2.5% (w/v) nonfat dry milk. After three 5-minute washes with TBST, the appropriate peroxidase-linked secondary antibody diluted 1:2,000 in TBST with 2.5% (w/v) nonfat dry milk was added and incubated for 1 hour at room temperature.

After a final washing step, four 5-minute washes with TBST, the proteins were detected using chemiluminescence. For that, the two ECL<sup>®</sup> solutions of the ECL<sup>®</sup> detection system were mixed in equal quantities (2 + 2ml for an 8 x 10cm membrane) and added to the moist membrane for 1 minute. The blot was immediately exposed to a film for approximately 60 seconds and developed. This method allows „stripping“ the membrane up to three times and performing different immunodetections on the same blot. In this study, each blot was stripped and re-blotted with an anti  $\beta$ -tubulin antibody in order to control for even protein loading. For stripping, the membrane was rotated in the waterbath at 55°C for 30 minutes in stripping solution (see buffers), rinsed 1x with TBST, blocked for 2 hours and incubated with the primary antibody as described above.

#### SAPK/JNK ASSAY KIT

We employed a non-radioactive method using the SAPK/JNK assay kit to measure cJun phosphorylating activity at residues Ser63 and Ser73 in c-Jun, which is generally JNK specific. 250 $\mu$ g protein of whole cell lysates was incubated overnight with N-terminal c-Jun (amino acids 1-89) fusion protein bound to glutathione sepharose (GST) beads to pull down JNK. Amino acids 1-89 in c-Jun contain a high-affinity binding site for JNK just N-terminal to the Ser63 and Ser73 phosphorylation sites. The beads were washed twice with a provided cell lysis buffer (SAPK/JNK assay kit), followed by two washes with a provided kinase buffer (SAPK/JNK assay kit). The kinase reaction was carried out during a 30-minute incubation period at 30°C in the presence of non-radioactive ATP. The reaction was terminated with 25 $\mu$ l of 2x SDS-PAGE gel loading dye (see buffers) and c-Jun phosphorylation was measured by Western blot using an antibody against phosphorylated Ser63 in c-Jun, which is usually JNK specific.

Using this method, the possibility cannot be ruled out that kinases other than JNK phosphorylate Ser63 in c-Jun. The serine/threonine kinase c-Raf-1/c-mil, for example, has been shown to phosphorylate Ser63 in c-Jun in a JNK independent manner. For verification, we confirmed the results obtained by the SAPK/JNK assay kit by directly measuring phosphorylated Thr183/Tyr185 in JNK by Western blot.

To rule out direct binding of JNK to GST, lysates were incubated with GST beads alone. Blots were stripped and reblotted with a GST antibody to control for variability in gel loading.

## 4.8 Electrophoretic mobility shift assay (EMSA)

### PREPARATION OF DOUBLE-STRANDED OLIGONUCLEOTIDES

20 $\mu$ g of two complementary oligonucleotides were diluted in a total volume of 100 $\mu$ l of 1x annealing buffer (see buffers). The oligonucleotide mix was heated to 95°C for 5 minutes and slowly cooled down in order to anneal the complementary oligonucleotides (for example by switching off the water bath and allowing to cool overnight). Double-stranded oligonucleotides were stored at -20°C.

### LABELING OF THE PROBE

10 $\mu$ l of double-stranded oligonucleotides were diluted in 70 $\mu$ l of 1x annealing buffer to make a 50ng/ $\mu$ l oligonucleotide stock. In 20 $\mu$ l labeling reaction, 1 $\mu$ l of the 50ng/ $\mu$ l oligonucleotide stock was mixed with 2 $\mu$ l of 10x polynucleotide kinase buffer, 5 $\mu$ l of 10mCi/ml [ $\gamma$ -<sup>32</sup>P]ATP (3000Ci/mmol), 2 $\mu$ l of polynucleotide kinase (10,000U/ml) and ddH<sub>2</sub>O. The reaction was incubated for 1 hour at 37°C. To remove un-incorporated oligonucleotides, the labeled oligonucleotides were purified using QuickSpin<sup>®</sup> columns. For that, the columns were pre-centrifuged at 1,000 rpm for 2 minutes to remove the buffer and then 20 $\mu$ l of labeled oligonucleotides were added to the column and spun for 5 minutes at 2,800 rpm. The labeled oligonucleotides were stored in a lead box at -20°C.

#### GELSHIFT ASSAY

A 5.2% polyacrylamide gel (see buffers) was prepared and polymerised for 20 to 30 minutes. The wells were rinsed with a 1ml pipette and the gel was pre-run for 30 minutes at 140 volts. Meanwhile, the 20 $\mu$ l binding reaction was prepared in the following sequence: 4 $\mu$ l of 5x binding buffer (see buffers) > 2 $\mu$ l of poly(dI-dC) as a non-specific competitor DNA > ddH<sub>2</sub>O > Protein > 200 fold molar excess (100ng) of unlabeled competitor oligonucleotides > 1 $\mu$ l of labeled probe. As a protein source, we used either 5 $\mu$ g of nuclear extracts or 3 $\mu$ l of *in vitro* translated protein or unprogrammed reticulocyte lysate. The reaction was incubated for 30 minutes at room temperature. For supershift experiments, 3 $\mu$ l of 200 $\mu$ g IgG/0.1ml antibody was added and incubated for another 30 minutes at room temperature. Reactions were electrophoresed at 130 volts at 4°C, dried and placed on x-ray film at -80°C with an intensifying screen.

#### 4.9 AnnexinV apoptosis assay

In the early apoptotic process, phospholipid asymmetry is disrupted leading to the exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane. AnnexinV is an anticoagulant protein that preferentially binds negatively charged phospholipids. We used AnnexinV-FITC conjugates for rapid detection of apoptotic cells by flow cytometry. Cells were washed twice with PBS containing 2% of FBS at 1,600 rpm for 5 minutes and softly vortexed in between. Then, 2 $\mu$ l of 50 $\mu$ g/ml AnnexinV-FITC and 2 $\mu$ l of 50 $\mu$ g/ml propidium iodide were added in a total volume of 100 $\mu$ l of AnnexinV binding buffer and incubated for 20 minutes in the dark.

#### ANALYSIS BY FLOW CYTOMETRY

10,000 cells were analyzed by flow cytometry. The X-axis of the dot blot reflected the logarithmic AnnexinV-FITC fluorescence and the Y-axis the propidium iodide fluorescence. Thus, unlabeled viable cells appeared in the lower left quadrant, early apoptotic cells that have bound AnnexinV in the lower right quadrant and late apoptotic or necrotic cells that have taken up the propidium iodide appeared in the upper right quadrant of the dot blot. We compared the percentage of AnnexinV-FITC positive cells.

#### 4.10 Statistical analysis

To compare the median of two groups, we performed the Mann-Whitney Wilcoxon test. In this study, we compared the absolute signal intensities for c-jun expression analyzed by microarray analysis (Affymetrix chips). The signal intensity for c-jun expression in each of the most frequent AML-associated translocations was compared to c-jun expression in normal bone marrow cells and the differences analyzed for their statistical significance. A p-value =0.05 indicated statistically significant differences between two groups.

In order to analyze whether there is a positive correlation between two factors within the same group, in our case between the mRNA expression of c-jun and the mRNA expression of AML1-ETO in t(8;21) positive patient samples, we calculated a Pearson correlation coefficient (r). r represents the deviation of two factors from a regression line. r=1 reflects the optimum, in the case of two factors lying directly on the regression line. The respective p-value for r was calculated for statistical significance. A p-value =0.05 indicated statistical significance.

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## 5. RESULTS

### 5.1. High c-jun mRNA expression levels in leukemia patient cells with t(8;21), t(15;17) or inv(16), and correlative mRNA expression levels for AML1-ETO and c-jun within t(8;21) positive leukemia patient cells

CML leukemia cells with the translocation t(9;22) have previously been associated with increased c-jun expression. Thus, we examined whether mRNA expression levels for c-jun are also elevated in AML leukemia cells with the most common cytogenetic abnormalities. Microarray analysis was performed of AML patient cells with translocation t(8;21), t(15;17), inv(16), translocations involving the mixed lineage leukemia (MLL) gene on chromosome 11q23: t(11q23/MLL), or of normal bone marrow cells, in a co-operation with the Leukemia Diagnostic Department, Klinikum Großhadern, Munich.

In the present study this data was statistically analyzed for the expression of human c-jun. We found that in normal bone marrow cells, c-jun mRNA expression levels were consistent with a standard deviation of 18.7 (Figure 1). Therefore, c-jun mRNA expression levels in normal bone marrow cells served as a reference point to which we compared the mean and standard deviations of c-jun mRNA expression levels in leukemia patient cells. We found increased expression of c-jun mRNA in t(8;21), t(15;17) or inv(16) positive primary leukemia patient cells. This increase was statistically significant when compared to expression levels of c-jun mRNA in normal bone marrow cells (Figure 1). There was, however, no statistically significant change between c-jun mRNA expression levels in t(11q23/MLL) positive leukemia patient cells and normal bone marrow cells, indicating that t(11q23/MLL) positive patient cells had basal levels of c-jun mRNA expression. CML patient cells with t(9;22) were included as positive controls. These had significantly higher c-jun mRNA expression levels (Figure 1).

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Standard deviations of c-jun mRNA expression levels in t(8;21), t(15;17) or inv(16) positive leukemia patient cells were about 4 times higher compared to normal bone marrow cells (Figure 1). In the case of t(8;21), for which the standard deviation was highest, we therefore investigated whether the high variation in c-jun mRNA expression levels was a consequence of fluctuating mRNA expression levels of AML1-ETO.

We performed real-time PCR for AML1-ETO (Figure 2A) and c-jun (Figure 2B) of 16 t(8;21) positive patient samples and found a 13 fold variation between the mRNA expression levels of AML1-ETO and c-jun. In order to determine whether these variations were correlated, the average ratios of c-jun (Figure 2B) and AML1-ETO (Figure 2C) mRNA expression levels were compared by correlation analysis. We found statistically significant correlation between c-jun and AML1-ETO mRNA expression levels within t(8;21) patient samples with a p-value =0.01 (Figure 2C).

This data indicates that t(8;21), t(15;17) or inv(16) positive leukemic AML patient cells contain significantly higher mRNA expression levels of c-jun than normal bone marrow cells. Furthermore, within t(8;21) positive AML patient cells, the mRNA expression levels of AML1-ETO and c-jun are correlated.

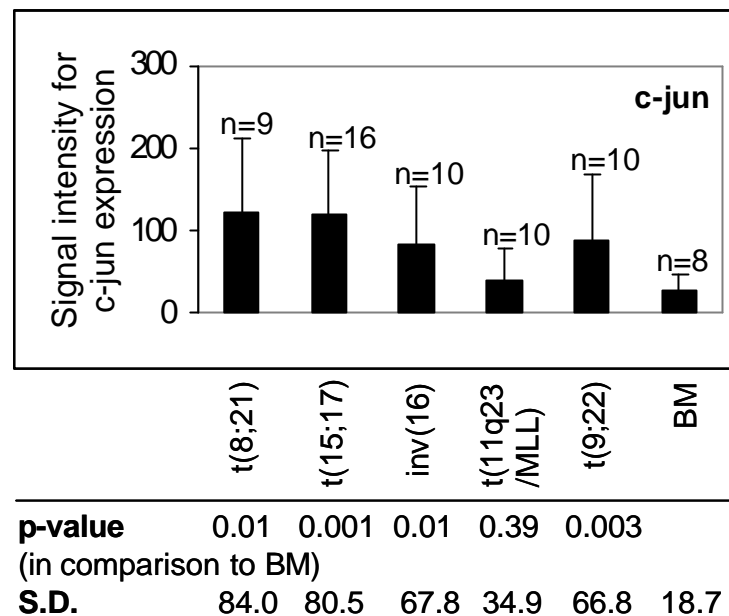
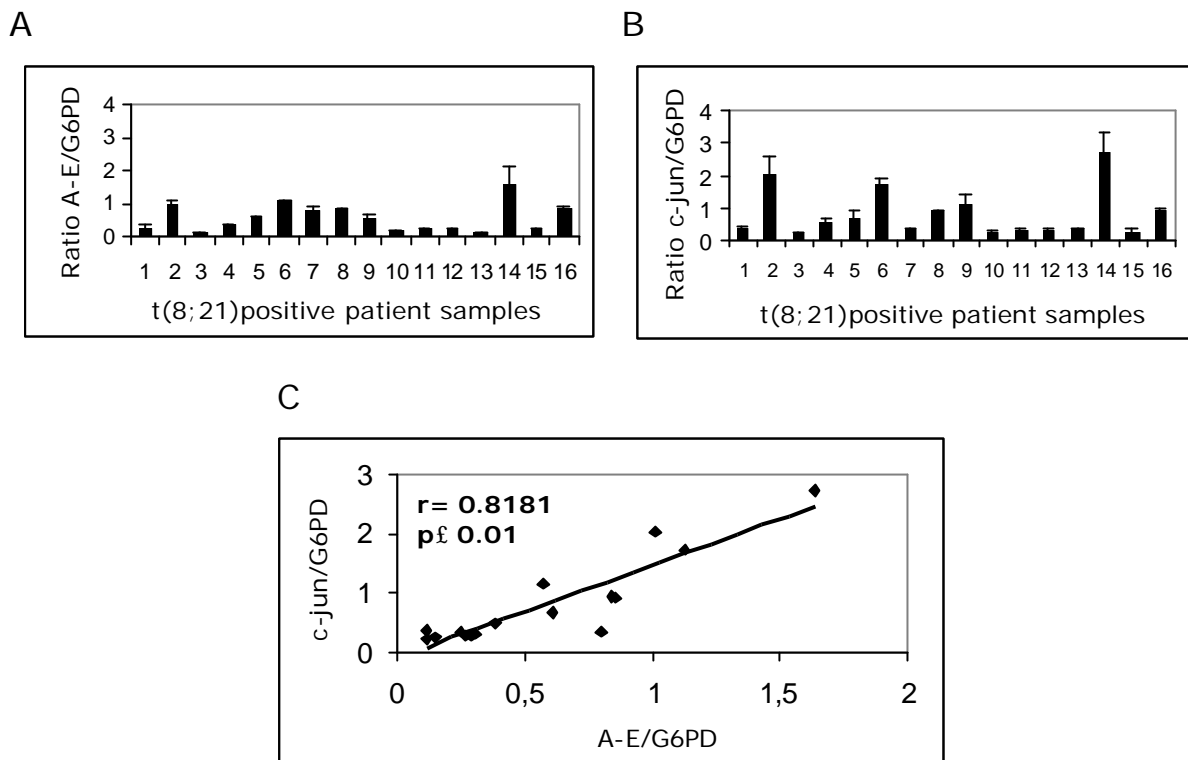


Figure 1: **High c-jun mRNA expression in leukemia patient cells with t(8;21), t(15;17), inv(16) or t(9;22) compared to normal bone marrow cells.**

Comparison of absolute signal intensities for c-jun mRNA expression from microarray analysis (Affymetrix) of leukemia patient cells with t(8;21), t(15;17), inv(16), t(9;22), or t(11q23/MLL) to normal bone marrow (BM) cells. Number of patients analyzed (n) and the standard deviation (S.D.) is given for each group. The median c-jun expression of these leukemic translocations was statistically compared to c-jun expression in normal bone marrow cells using the Mann-Whitney Wilcoxon test. A p-value  $\leq 0.05$  indicates statistical significance.



**Figure 2: Highly significant correlation between c-jun and AML1-ETO mRNA expression levels within t(8;21) positive AML cells.**

(A) Real-time PCR for AML1-ETO (A-E) and G6PD of 16 t(8;21) positive AML patient samples. Ratios for A-E/G6PD were compared to each other. Data is shown as mean  $\pm$  standard deviation (S.D.) of 2 different experiments. Patient numbers 3, 5, 6, 8, 10, 12 and 15 have only been analyzed once for the expression of A-E/G6PD.

(B) Real-time PCR for c-jun of the same patient samples as described in (A). Ratios of c-jun/G6PD are given. Data is shown as mean  $\pm$  S.D. of 2 different experiments.

(C) Correlation analysis: Average ratios for c-jun/G6PD and A-E/G6PD expression of 16 t(8;21) positive AML patient samples are plotted as an xy scatter plot. Correlation coefficient ( $r$ ) and  $p$ -value are shown. A  $p$ -value  $\leq 0.05$  indicates statistical significance.



## 5.2 AML1-ETO induction increases c-jun mRNA and c-Jun protein expression

To demonstrate that AML1-ETO itself is responsible for the increased c-jun mRNA expression levels in t(8;21) positive patients samples, we used a myeloblastic U937 cell line with AML1-ETO under the control of a zinc-inducible metallothionein promoter (U937Z/A-E) and performed quantitative real-time PCR for AML1-ETO and c-jun at different time points of AML1-ETO induction. In this inducible system, AML1-ETO was maximally expressed (6 fold) after 2 hours of  $Zn^{2+}$ -induction and slowly decreased thereafter (Figure 3A, upper panel). As shown in Figure 3A, middle panel, AML1-ETO expression induced an approximately 5 fold increase in c-jun expression after 4 and 6 hours of  $Zn^{2+}$ -induction and c-jun levels remained elevated at 16 hours of induction. The addition of  $Zn^{2+}$  did not affect c-jun expression in control parental U937 cells (Figure 3A, lower panel).

To investigate whether c-Jun protein levels are also affected upon AML1-ETO induction, Western blot experiments were performed using a tetracycline-regulated AML1-ETO expressing U937 cell line (U937T/A-E). This cell line expresses high and tightly controlled levels of AML1-ETO protein upon tetracycline withdrawal that can be easily quantified by Western blot analysis (Figure 3B, upper panel).<sup>88</sup> c-Jun protein expression increased between 24 and 48 hours of AML1-ETO induction in the U937T/A-E cell line (Figure 3B, middle panel), whereas c-Jun expression remained unchanged in the U937T control cell line (Figure 3B, lower panel).

Thus, expression analysis by real-time PCR or Western blot using two different inducible systems for AML1-ETO, clearly showed upregulation of c-jun mRNA or c-Jun protein expression, respectively, upon AML1-ETO induction in myeloid U937 cells.

Figure 3A

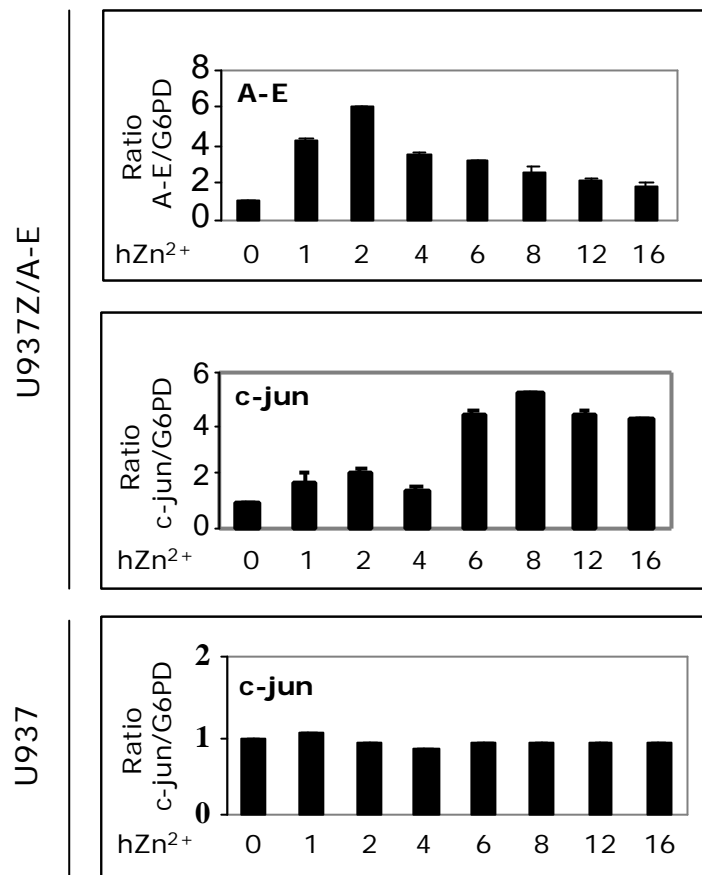
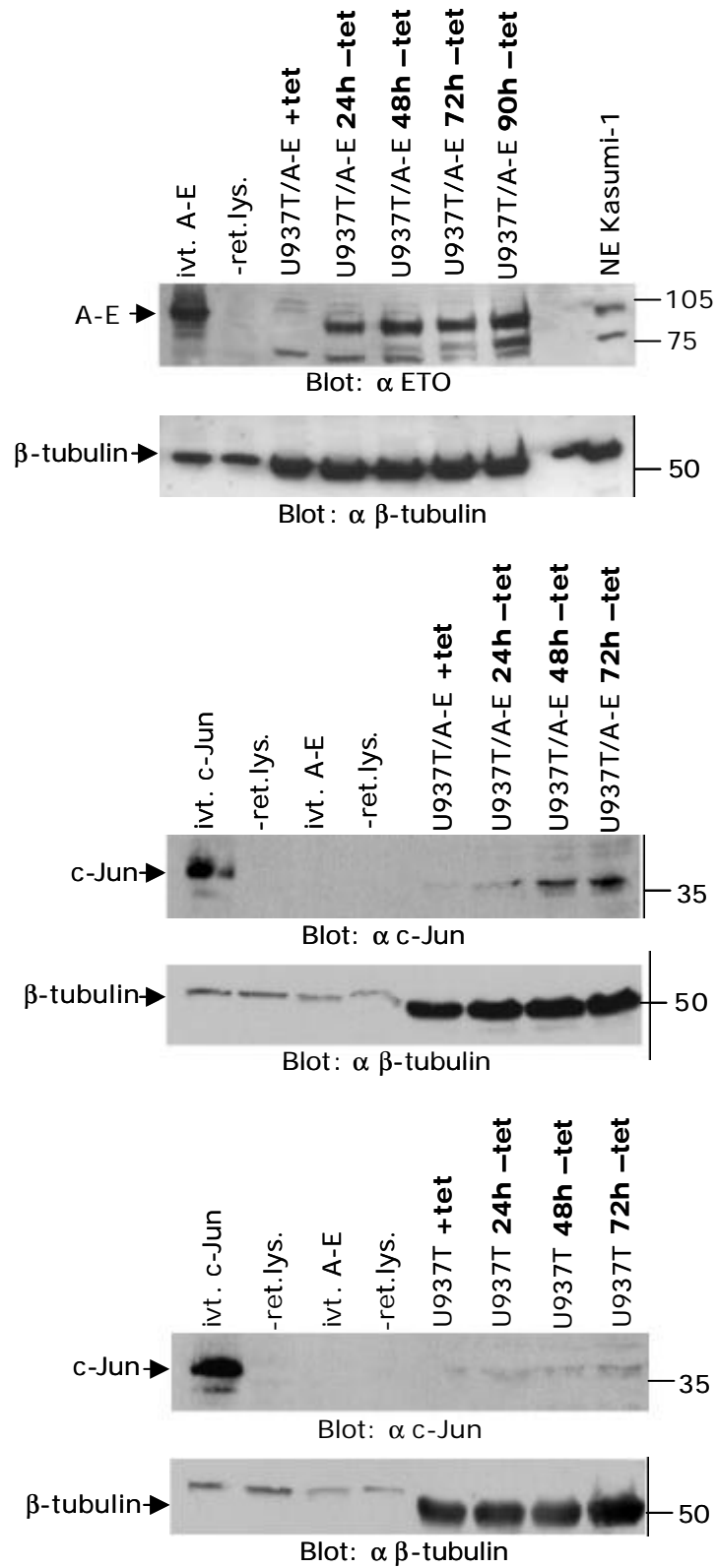


Figure 3A: **Upregulation of c-jun mRNA expression upon AML1-ETO induction.**

Real-time PCR analysis for AML1-ETO (A-E) and c-jun. U937Z/A-E and U937 cells were washed twice in phosphate-buffered saline (PBS) and stimulated with  $100\mu\text{M Zn}^{2+}$  for 1, 2, 4, 6, 8, 12 or 16 hours (h Zn<sup>2+</sup>). cDNA was prepared and tested for expression of c-jun, AML1-ETO (A-E) and housekeeping gene G6PD using real-time PCR technology. Ratios of c-jun/G6PD (middle panel) and A-E/G6PD (upper panel) are shown as fold upregulation compared to time point zero (no addition of Zn<sup>2+</sup>), which was set to 1 fold. Wildtype U937 cells were analyzed for c-jun expression to control for Zn<sup>2+</sup> effects (lower panel). Data is shown as mean  $\pm$  S.D. of 3 different experiments.

Figure 3B



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**Figure 3B: Upregulation of c-Jun protein expression upon AML1-ETO induction.**

Western blot analysis using whole cell lysates of U937T/A-E and U937T cell lines. Cells were washed three times with PBS and grown for 24, 48, 72 or 90 hours in the maintenance medium with tetracycline (+tet: no AML1-ETO expression), or without tetracycline (-tet: AML1-ETO expression). Western blot analysis for AML1-ETO using an anti ETO antibody (a ETO) of U937T/A-E cell lysates is shown in the upper panel. Kasumi-1 cell lysates containing AML1-ETO were included as positive controls. The middle panel shows the same U937T/A-E cell lysates examined for expression of c-Jun. In the lower panel, the empty vector U937T cell lysates were blotted for c-Jun to control for tet-off effects on c-Jun protein expression. *In vitro* translated (*ivt.*) AML1-ETO (A-E) and c-Jun proteins were included as controls in order to verify protein sizes. Reticulocyte lysate (ret. lys.) represents a negative control for *in vitro* translation containing only reticulocyte lysate. Every blot was stripped and reblotted for  $\beta$ -tubulin as a loading control.

### 5.3 AML1-ETO enhances the positive autoregulatory activity of c-Jun by transactivating the human c-jun promoter through the proximal AP-1 site

Next, we studied whether the increased level of c-Jun protein upon AML1-ETO expression resulted from direct stimulation of the human c-jun promoter. Furthermore, it was of interest to compare the transactivating capacity of AML1-ETO to wildtype AML1 and ETO protein. Thus, we transiently transfected U937 cells with the full-length human c-jun promoter controlling the luciferase reporter gene and co-transfected AML1-ETO, AML1 or ETO. AML1 alone has been shown to be a weak transactivator, therefore, we also co-transfected AML1 and its co-activator CBF $\beta$  together, which have been shown to co-operatively upregulate the M-CSF receptor promoter<sup>104</sup> (Figure 4B, lower panel, right).

AML1-ETO transactivated the full-length human c-jun promoter around 3 fold, whereas neither wildtype AML1, with or without CBF $\beta$ , nor wildtype ETO showed transactivation capacity (Figure 4B, upper panel). A minimal promoter for NF- $\kappa$ B fused to the luciferase gene, which was not transactivated upon AML1-ETO, served as a negative control (Figure 4B, lower panel, left). Since ETO has not been described to be capable of DNA binding, there was no positive control available.

Thus, we concluded that AML1-ETO transactivation capacity on the c-jun promoter is an acquired function of the fusion protein.

We next went on mapping the site in the c-jun promoter being transactivated by AML1-ETO by transiently transfecting various 5' c-jun promoter deletion mutants<sup>11</sup> into U937 cells. The site in the c-jun promoter being transactivated by AML1-ETO could be narrowed down to a region between bp -180 to bp +731 (around 3.5 fold upregulation of c-jun promoter activity) and bp -63 to bp +731 (upregulation of c-jun promoter activity was lost) (Figure 4C).

Interestingly, in the longer c-jun promoter constructs ranging from bp -1780 to bp -345/+731, transactivation capacity of AML1-ETO decreased up to 1.5 fold, which implies loss of positive response elements (Figure 4C). The increase in c-jun activation by AML1-ETO upon removal of the sequence from bp -345 to bp -180 suggests the presence of a repressive element (possibly the upstream AP-1 site). The bp -180 to bp +731 c-jun promoter construct still contains the proximal AP-1 site (see Figure 4A).

Importance of the AP-1 site for AML1-ETO transactivation has previously been shown for a minimal AP-1 promoter derived from the collagenase gene in fibroblast cells.<sup>49</sup> Therefore, for fine mapping, we transiently transfected U937 cells with a c-jun promoter construct ranging from bp -79 to bp +170 or the same construct with the proximal AP-1 site mutated. Co-transfection of AML1-ETO transactivated the bp -79 to bp +170 c-jun promoter construct up to 4.5 fold. Transactivation capacity was reduced to 1.5 fold when the proximal AP-1 site was mutated and lost when we transfected a bp -63 to bp +170 construct which was included to rule out importance of the 3' promoter region between bp +170 and bp +731 (Figure 4D).

These results indicate that in U937 cells, AML1-ETO increased AP-1 activity of the c-jun promoter via the proximal AP-1 site.

Strikingly, the proximal AP-1 site is also the site to which c-Jun can feed back to autoregulate its expression. Thus, we hypothesized that AML1-ETO might enhance the autoregulatory capacity of c-Jun by increasing the amount of c-Jun protein. Therefore, we transiently transfected the bp -79 to bp +170 c-jun promoter construct and co-transfected c-jun, AML1-ETO or c-jun and AML1-ETO together into U937 cells, which contain only little endogenous c-Jun. AML1-ETO alone transactivated the c-jun promoter construct around 4 fold, c-jun transactivated its own promoter around 30 fold and upon co-transfection of AML1-ETO around 90 fold (Figure 4E).

These results suggest that c-jun and AML1-ETO co-operatively transactivate the c-jun promoter and that AML1-ETO enhanced the autoregulatory nature of c-jun.

Figure 4A

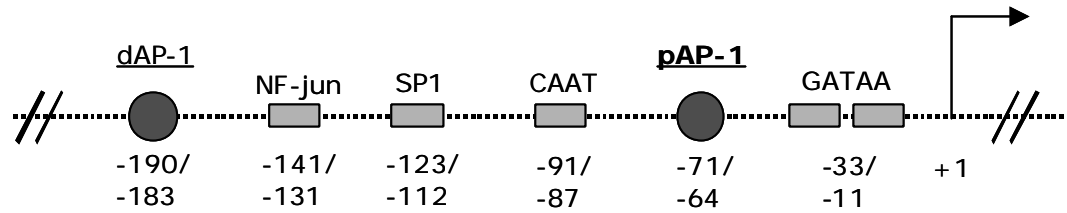
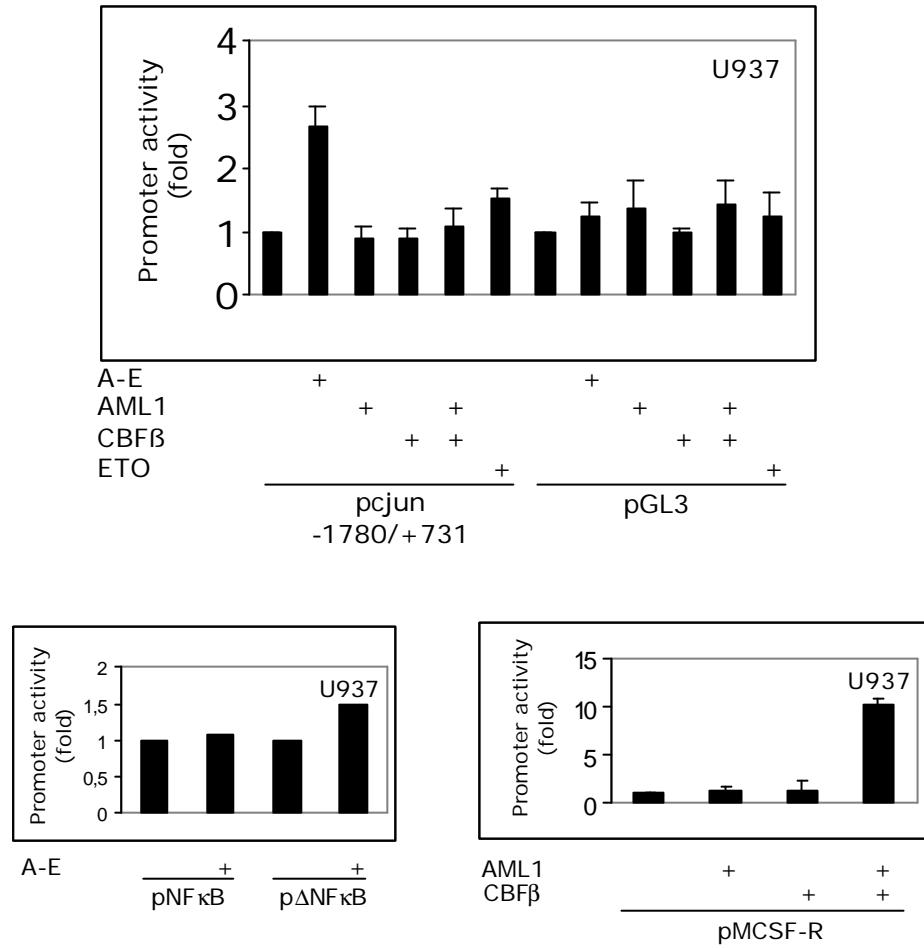


Figure 4A: Schematic representation of the proximal human c-jun promoter with important transcription factor binding sites in myeloid cells.

The proximal c-jun promoter contains 2 AP-1 sites, a proximal (pAP-1) and a distal (dAP-1) one, a CAAT binding site for CCAAT/enhancer binding proteins (C/EBPs) and binding sites for the transcription factors NF-jun, SP-1 and GATA-1.

Figure 4B





**Figure 4B: AML1-ETO but not wildtype AML1 or ETO transactivates the full-length human c-jun promoter.**

**Upper panel:** Effect of AML1-ETO, AML1, CBF $\beta$  or ETO on the transactivation capacity of the full-length human c-jun promoter (pcjun -1780/+731). U937 cells were transiently transfected with 0.6 $\mu$ g of the full-length human c-jun promoter or the promoterless vector pGL3. 0.05 $\mu$ g of the internal control plasmid pRL-null was added in all transfections. 0.2 $\mu$ g of expression plasmids for AML1-ETO (A-E), AML1, CBF $\beta$  or ETO in pCMV5, or empty vector alone were co-transfected and luciferase activities determined 30 hours after transfection. The normalized luciferase activity of each c-jun promoter construct was arbitrarily defined as 1 fold promoter activity. The promoter activity in presence of AML1-ETO was presented in relation to it (fold promoter activity). Thus, effects of AML1-ETO on the empty vector plasmid (pGL3) could be more visible. Transfection data is shown as mean  $\pm$  standard deviation (S.D.) of 3 separate experiments.

**Lower panel, left:** Negative control for the transactivation capacity of AML1-ETO (A-E). U937 cells were transiently transfected with 0.6 $\mu$ g of a minimal NF- $\kappa$ B promoter and the same construct with mutated NF- $\kappa$ B site. 0.2 $\mu$ g of A-E was co-transfected and the promoter activity determined. Promoter activity upon co-transfection of AML1-ETO was presented in relation to the promoter activity of the NF- $\kappa$ B promoter construct, which was arbitrarily set to 1 fold. Transfection data is shown as mean  $\pm$  S.D. of 3 separate experiments.

**Lower panel, right:** Positive control for the transactivation capacities of AML1 and CBF $\beta$ . The macrophage-colony stimulating factor receptor promoter (pMCSF-R) was transiently transfected into U937 cells and 0.2 $\mu$ g of AML1 and CBF $\beta$  was co-transfected. Data was presented as described above.

Figure 4C

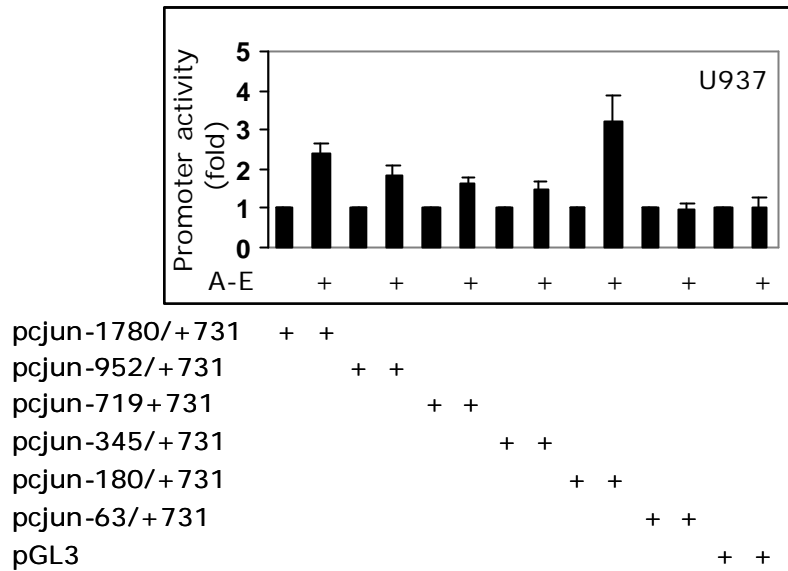
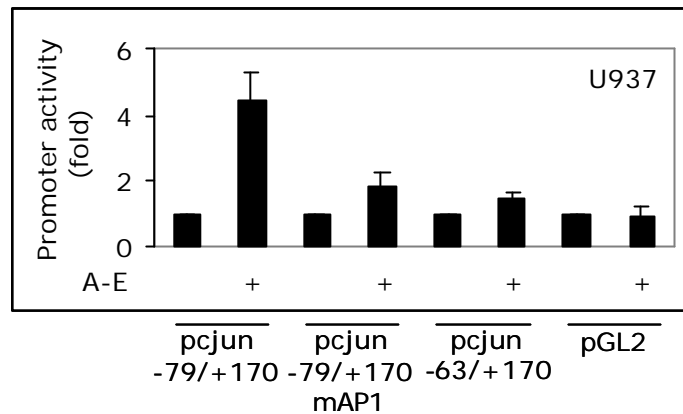


Figure 4C: Mapping of the AML1-ETO responsive site in the human c-jun promoter.

0.6 $\mu$ g of 5' truncation mutants of the human c-jun promoter (pcjun -1780, -952, -719, -345, -180, -63/+731) were transiently transfected into U937 cells and co-transfected with 0.2 $\mu$ g of pCMV5 AML1-ETO expression plasmid. The normalized luciferase activity of each c-jun promoter construct was arbitrarily defined as 1 fold. The promoter activity in presence of AML1-ETO was presented in relation to it (fold promoter activity). Transfection data is shown as mean  $\pm$  S.D. of 3 separate experiments.

Figure 4D



**Figure 4D: Fine mapping of the AML1-ETO responsive site in the proximal c-jun promoter.**

Transient transfection of U937 cells with 0.6 $\mu$ g of a proximal c-jun promoter construct (pcjun -79/+170) or the same construct with the proximal AP-1 site mutated (pcjun -79/+170 mAP-1), with a bp -63 to bp +170 c-jun promoter construct (pcjun -63/+170) or the empty vector pGL2. 0.1 $\mu$ g of the expression plasmid pCMV5 AML1-ETO (A-E) was co-transfected and promoter activity analyzed. The normalized luciferase activity of each c-jun promoter construct was arbitrarily defined as 1 fold. The promoter activity in presence of AML1-ETO was presented in relation to it (fold promoter activity). Transfection data is shown as mean  $\pm$  S.D. of 3 separate experiments.

Figure 4E

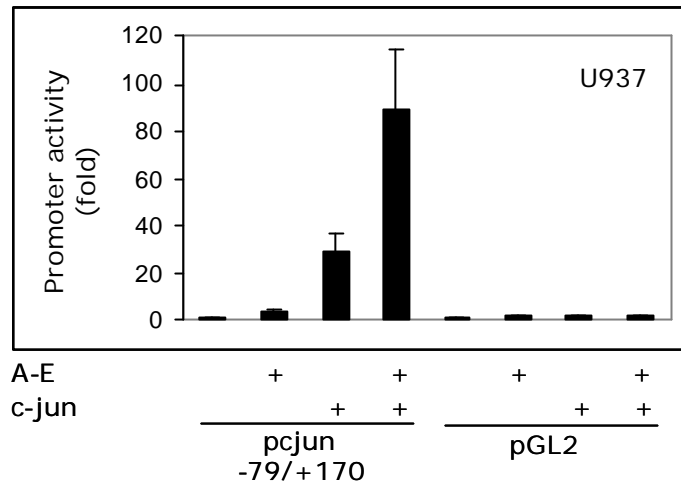


Figure 4E: **Co-operative transactivation of the c-jun promoter by AML1-ETO and c-jun.**

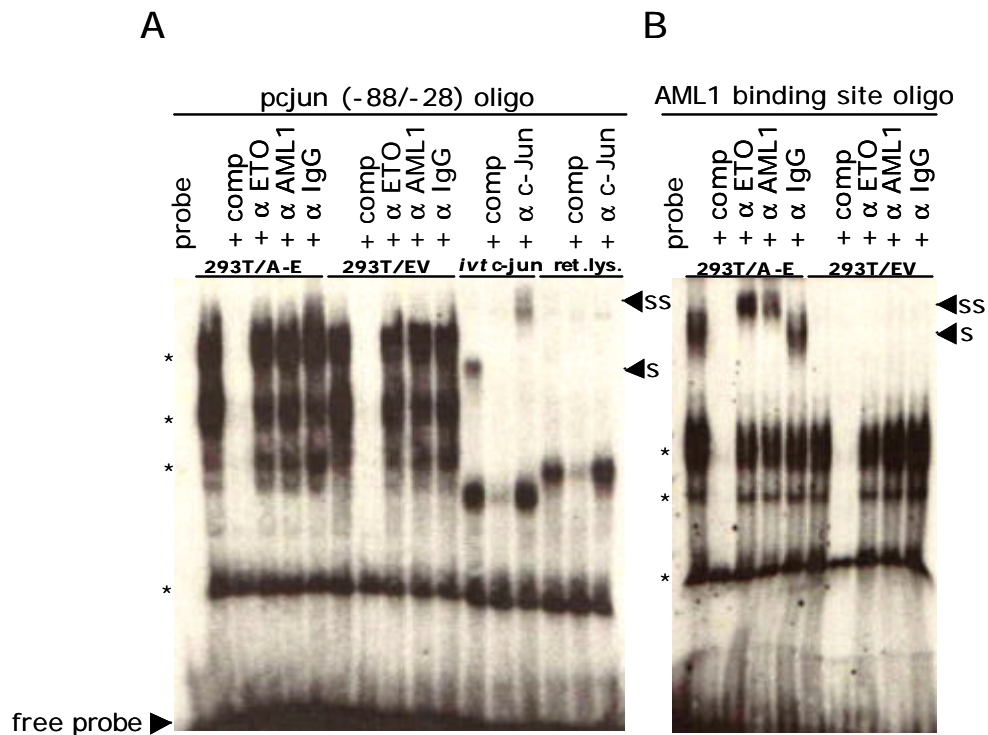
U937 cells were transiently transfected with 0.6 $\mu$ g of a proximal c-jun promoter construct (pcjun -79/+170) or the empty vector pGL2 and co-transfected with 0.1 $\mu$ g of AML1-ETO (A-E), c-jun, or A-E and c-jun together. Transfection was performed and the data analyzed as described above.

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#### 5.4 AML1-ETO cannot directly bind to the c-jun promoter

To investigate whether AML1-ETO enhanced AP-1 activity of the c-jun promoter by directly binding to DNA, we performed electrophoretic mobility shift assays (EMSA). As described in Figure 5A, nuclear extracts of AML1-ETO transfected 293T cells could not specifically bind to a c-jun promoter oligonucleotide ranging from bp -88 to bp -28: pcjun (-88/-28) oligo, but positively bound to a control AML1 consensus site oligonucleotide (AML1 binding site oligo) (Figure 5B). Along with these results, DNA binding of AML1-ETO has only been shown through the wildtype AML1 consensus site TG(T/c)GGT,<sup>62</sup> which is absent in the human c-jun promoter.

Thus, we concluded that AML1-ETO might transactivate the human c-jun promoter in an indirect manner.



**Figure 5: No physical binding of AML1-ETO to the proximal c-jun promoter.**

(A) Electrophoretic mobility shift assay (EMSA) with a proximal c-jun promoter oligonucleotide: pcjun (-88/-28) oligo. Oligonucleotide probes were incubated with 5 $\mu$ g of nuclear extracts of AML1-ETO transfected 293T cells (293T/A-E), or were mock transfected with the empty vector pCMV5 (293T/EV). 200 fold molar excess of unlabeled competitor self-probe (+comp) was added to the electrophoretic mobility shift assay. No specific supershifting of the band-shift complexes (non-specific bands were marked with an asterisk \*) were produced with anti ETO ( $\alpha$  ETO) or anti AML1 ( $\alpha$  AML1) antibodies. Anti IgG ( $\alpha$  IgG) antibody was added as an isotype control. *In vitro* translated c-Jun (*ivt* c-Jun) was used as a positive binding control to the c-jun promoter oligonucleotide and produced a specific shift (s) which could be competed away with 200x of competitor, was supershifted (ss) with c-Jun antibody and was absent in reticulocyte lysate (ret. lys.).

(B) EMSA as described above using an oligonucleotide containing an AML1 binding site (AML1 binding site oligo) as positive binding control for nuclear extracts of AML1-ETO transfected 293T cells (293T/A-E).

### 5.5 AML1-ETO induces the JNK signaling pathway

The c-jun promoter integrates several MAPK signaling pathways.<sup>105</sup> At the proximal AP-1 site, pre-bound c-Jun is mainly targeted by the JNK signaling pathway,<sup>106</sup> which increases transcriptional activity of c-Jun by phosphorylating Ser63 and Ser73 in c-Jun.<sup>17,18</sup> Therefore, the JNK signaling pathway might be involved in the indirect stimulation of the c-jun promoter by AML1-ETO.

We assayed the activity of JNK induced phosphorylation of Ser63 in c-Jun in U937T/A-E cells after 48 hours of AML1-ETO induction (U937T/A-E 48h -tet). U937T cells were included as a negative control (U937T 48h -tet). As shown in Figure 6A, phosphorylation of Ser63 in c-Jun was increased upon AML1-ETO induction in the AML1-ETO expressing cell lysates (lanes 3 and 4), whereas no effect on c-Jun phosphorylation was seen upon tetracycline withdrawal in the U937T control lysates (Figure 6A, lanes 1 and 2).

In order to rule out the possibility that kinases other than JNK phosphorylate Ser63 in c-Jun,<sup>107</sup> we directly measured the phosphorylation of JNK upon AML1-ETO induction. AML1-ETO induction for 48 hours increased phosphorylation of the 46 and the 54 kDa isoforms of JNK1 and JNK2,<sup>108</sup> and was maximally increased at 72 hours. There was no upregulation of JNK phosphorylation in the U937T control cell lysates (Figure 6B). To investigate whether AML1-ETO induction influenced the expression of JNK, we analyzed the same blot for JNK1 expression, which was not enhanced upon AML1-ETO induction (Figure 6B).

We further analyzed the phosphorylation of the endogenous JNK targets c-Jun and ATF-2 upon AML1-ETO expression. AML1-ETO induction for 48 and 72 hours increased JNK specific phosphorylation of Ser63 and Ser73 in c-Jun and Thr71 in ATF-2 by Western blot analysis (Figure 6C). There was a non-specific tetracycline-off effect on phosphorylation of c-Jun and ATF-2 at 24 hours in the U937T control cell line, which was, however, only transient and not visible at 48 and 72 hours of tetracycline withdrawal.

These results suggest that AML1-ETO activated the JNK pathway by inducing the phosphorylation of JNK and its targets c-Jun and ATF-2.



Figure 6A

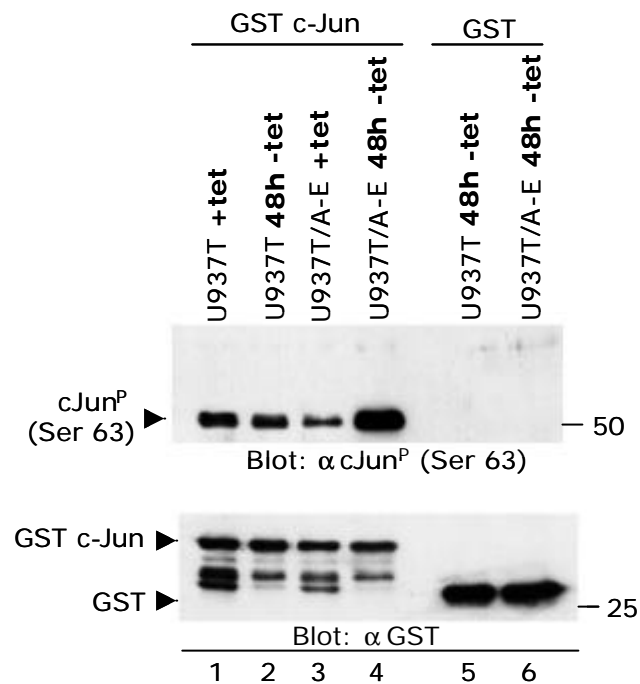


Figure 6A: AML1-ETO induces JNK specific activation of c-Jun.

*In vitro* kinase assay for JNK. Cell lysates of U937T and U937T/A-E cells, grown for 48 hours in the presence (+tet) or absence (48h -tet) of tetracycline, were incubated with GST N-terminal c-Jun (amino acids 1-89) fusion beads (lanes 1-4) or GST beads alone (lanes 5 and 6). Kinase reaction was triggered and indirectly analyzed by performing Western blot for JNK dependent phosphorylation of Ser63 in c-Jun: c-Jun<sup>P</sup> (Ser63). As a control for even protein loading, the same blot was stripped and reblotted with a GST antibody.

Figure 6B

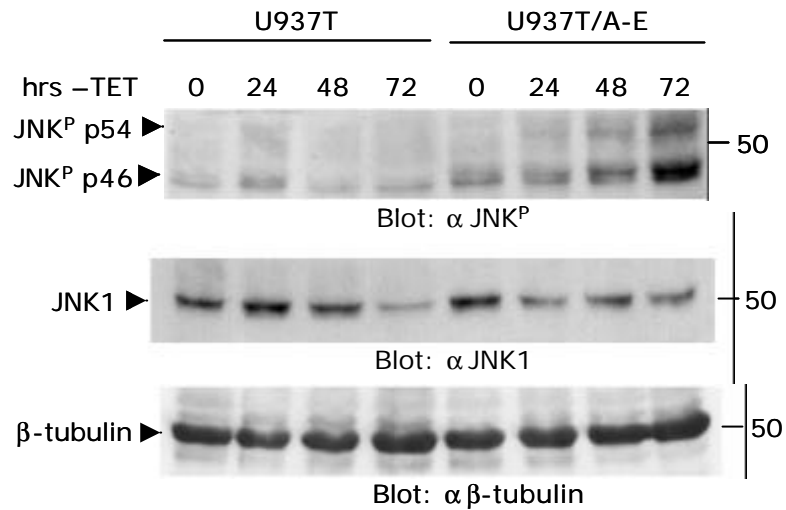


Figure 6B: AML1-ETO induces phosphorylation of JNK.

Western blot using an antibody against phosphorylated Thr183 and Tyr185 in JNK, which recognizes JNK isoforms of 46 and 54 kDa (JNK<sup>P</sup> p46, JNK<sup>P</sup> p54). Cell lysates of U937T and U937T/A-E cells were grown with tetracycline, or for 24, 48, or 72 hours without tetracycline. Blots were stripped and reblotted with an anti JNK1 antibody (α JNK1) to determine JNK1 expression, stripped again and reblotted for β-tubulin as loading control.

Figure 6C

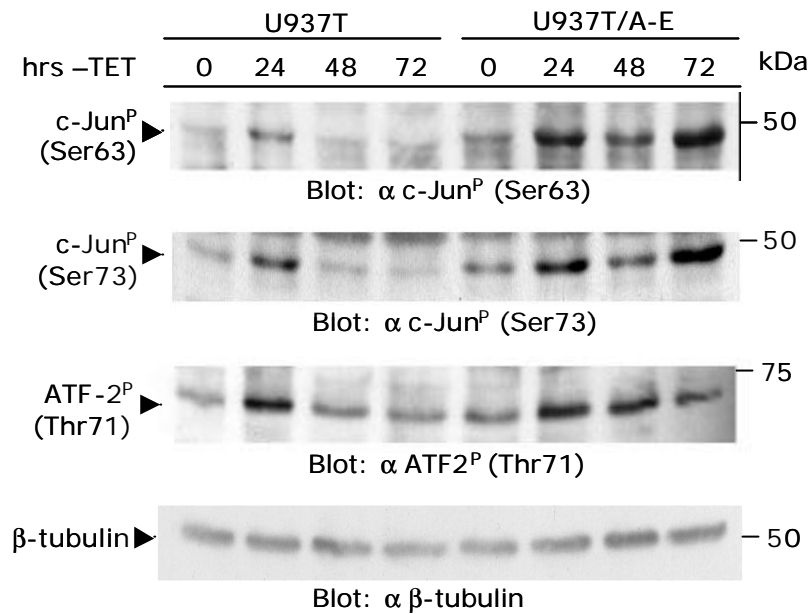


Figure 6C: **AML1-ETO induces phosphorylation of the JNK targets c-Jun and ATF-2.**

Western blot for phosphorylated Ser63 in c-Jun: c-Jun<sup>P</sup> (Ser63), phosphorylated Ser73 in c-jun: cjun<sup>P</sup> (Ser73) and for phosphorylated Thr71 in ATF-2: ATF-2<sup>P</sup> (Thr71) using cell lysates of U937T and U937T/A-E cells grown for 24, 48 or 72 hours in the presence or in the absence of tetracycline. Each blot was re-blotted for β-tubulin to control for protein loading.

## 5.6 Increased DNA binding of c-Jun and ATF-2 to the proximal AP-1 site of the c-jun promoter upon AML1-ETO induction

The proximal AP-1 site is preferentially occupied by the AP-1 transcription factors and JNK targets c-Jun and ATF-2, which were both phosphorylated upon AML1-ETO induction. Therefore, we investigated by EMSA whether c-Jun and ATF-2 also bind to the proximal AP-1 site of the c-jun promoter in U937 cells and whether AML1-ETO induction has any effect on AP-1 composition or DNA binding capacity.

Nuclear extracts of U937T/A-E or U937T cells, grown for 48 hours under tetracycline removal, served as a protein source. EMSA revealed that in U937 cells, c-Jun and ATF-2 could bind to the proximal AP-1 site of the c-jun promoter, but not c-fos. Binding to the proximal AP-1 site was confirmed by performing EMSA using a c-jun promoter oligonucleotide with mutated AP-1 site. AML1-ETO induction for 48 hours in the U937T/A-E cells (U937T/A-E) did not change the composition of AP-1 factors binding to the proximal AP-1 site compared to nuclear extracts of U937T control cells (Figure 7A). However, EMSA with increasing concentrations of specific competitor DNA revealed that upon AML1-ETO expression, the DNA binding of c-Jun and ATF-2 to the c-jun promoter was increased (Figure 7B).

These results indicate that in U937 cells c-Jun and ATF-2 can bind to the proximal AP-1 site of the c-jun promoter. Thus, via phosphorylating c-Jun and ATF-2, the JNK signaling pathway might connect AML1-ETO to the proximal AP-1 site of the c-jun promoter. Furthermore, via stimulating the JNK pathway, AML1-ETO enhanced the DNA binding capacity of c-Jun and ATF-2 to the proximal AP-1 site, which might result in enhanced c-jun promoter activity.

Figure 7A

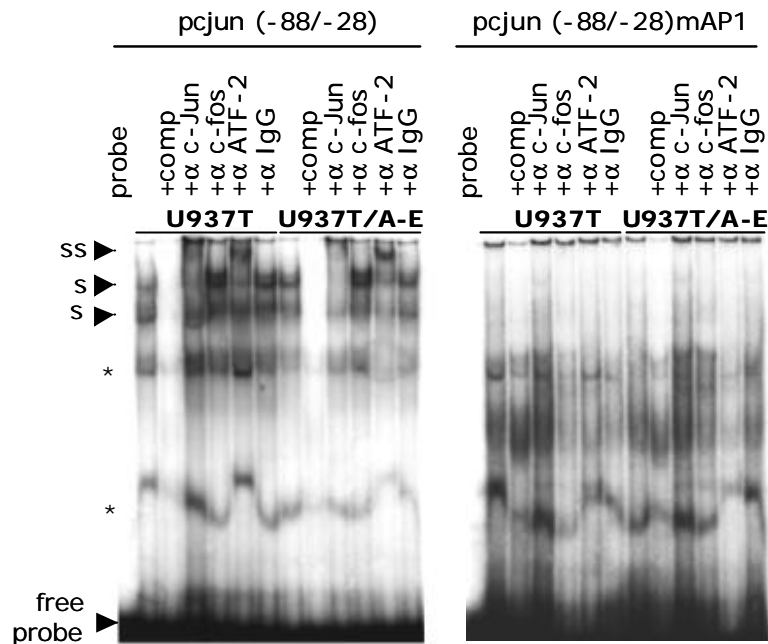
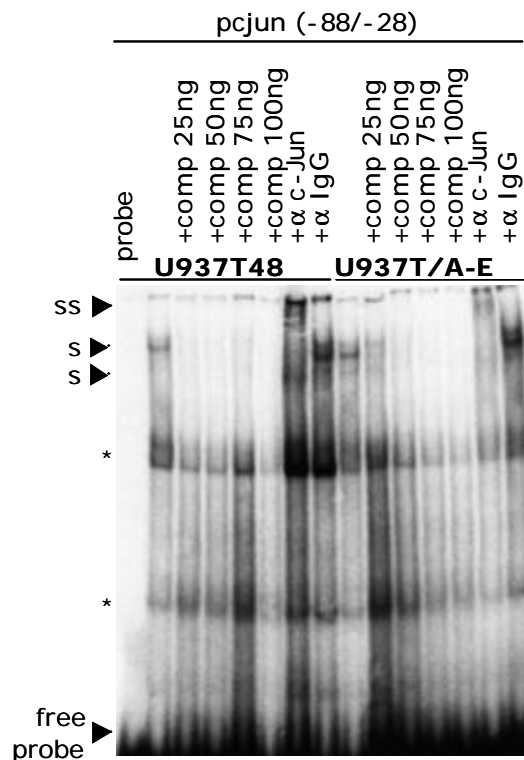


Figure 7A: c-Jun and ATF-2 bind to the proximal AP-1 site in U937 cells.

EMSA using a bp -88 to bp -28 c-jun promoter oligonucleotide: pcjun (-88/-28) (**left panel**) or a bp -88 to bp -28 c-jun promoter oligonucleotide with mutated AP-1 site: pcjun (-88/-28) mAP-1 (**right panel**). Nuclear extracts of U937T cells (U937T) and U937T/A-E cells (U937T/A-E), both grown under tetracycline withdrawal for 48 hours, were compared. Equal quantities of nuclear extracts were added to the binding reaction and produced a doubled-banded shift, of which the slower migrating band was supershifted with c-Jun or ATF-2 antibodies, but not with antibodies against c-fos. The faster-migrating band was supershifted neither with c-Jun nor with ATF-2 or c-fos antibodies. Specificity of the band-shifts was established by adding 200 fold excess of non-radioactive pcjun (-88/-28) self-probe (+comp).  $\alpha$  IgG rabbit was used as isotype control. EMSA using pcjun (-88/-28) mAP-1 as a probe failed to bind c-Jun or ATF-2. Asterisks (\*) indicate unspecific shifts.

Figure 7B



**Figure 7B: Increased DNA binding of c-Jun and ATF-2 upon AML1-ETO induction.**

EMSA using a bp -88 to bp -28 c-jun promoter oligonucleotide: pcjun (-88/-28), as described in Figure 7A. Increasing concentrations of non-radioactive pcjun (-88/-28) self-probe (25ng to 100ng) were added in order to slowly compete away the slower migrating shift composed of c-Jun and ATF-2. Asterisks (\*) indicate unspecific shifts.

## 5.7 Interference with the JNK signaling pathway disrupts the functional properties of AML1-ETO

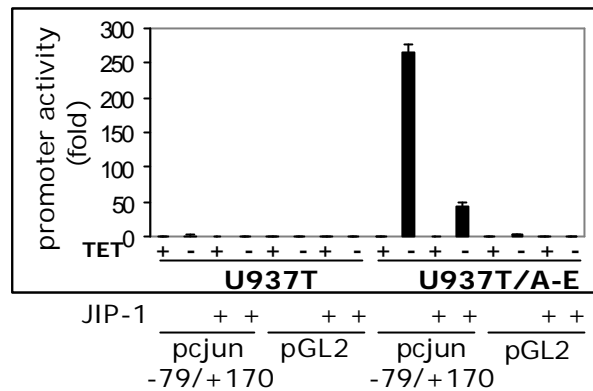
In order to elucidate the functional importance of the JNK signaling pathway for AML1-ETO induced transactivation of the c-jun promoter, we investigated whether disruption of the JNK signaling pathway can interfere with the transactivation capacity of AML1-ETO on the c-jun promoter (Figure 8A).

For that, we transiently transfected a proximal c-jun promoter construct (bp -79 to bp +170) into U937T/A-E and U937T cells, each grown with or without tetracycline and overexpressed JIP-1. JIP-1 is a scaffold protein which aggregates components of the JNK pathway to a functional signaling module.<sup>109</sup> In overexpression studies, however, JIP-1 sequesters JNK from its nuclear targets,<sup>110</sup> like c-Jun and ATF-2, and thus functions as an inhibitor of JNK regulated gene expression.<sup>111</sup> Overexpression of JIP-1 in U937T/A-E cells after 36 hours of AML1-ETO induction, reduced transactivation capacity of AML1-ETO approximately 5 fold, from around 260 to 40 fold, which suggests that JNK might play a role in the stimulation of the c-jun promoter by AML1-ETO.

Furthermore, we assessed whether the inhibition of JNK could revert the biological function of AML1-ETO. AML1-ETO induction for 3 days in U937 cells has been shown to induce proliferation arrest and apoptosis.<sup>88</sup> We tried to block AML1-ETO induced apoptosis with cell-permeable JNK specific inhibitor peptides (JNK inh.).<sup>102</sup> As revealed in AnnexinV assay, addition of these peptides to U937T/A-E cells after 72 hours of AML1-ETO induction reduced AML1-ETO induced apoptosis by around 30%, while addition of the HIV-TAT 48-57 negative control peptide (neg.co.) had no effect. No effect was seen for U937T/A-E cells grown in the presence of tetracycline or for the U937T control cell line (Figure 8B).

These results underline the biological importance of the JNK signaling pathway for AML1-ETO induced apoptosis in U937 cells.

Figure 8A



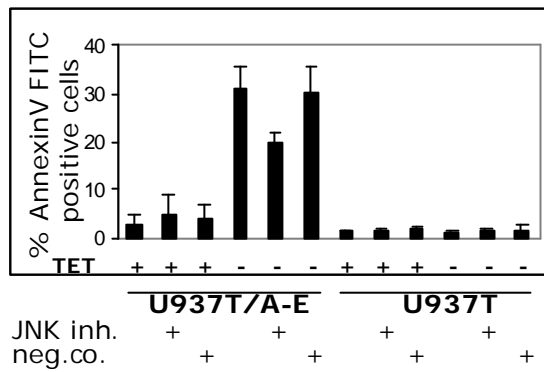
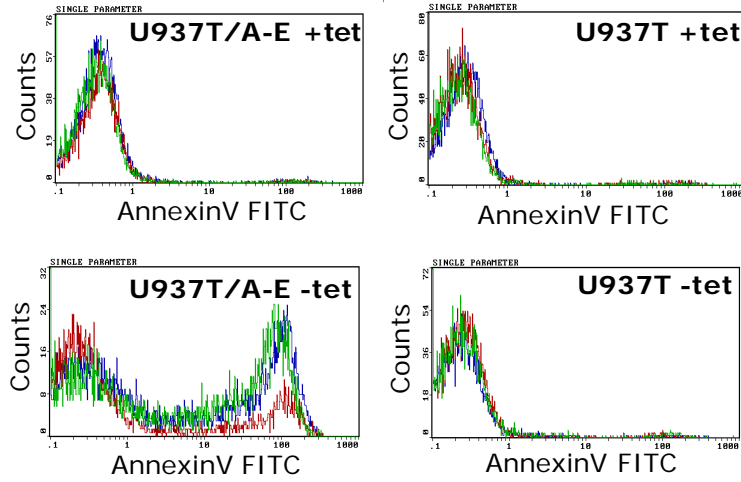
**Figure 8A: Disruption of the JNK signaling pathway reduces the transactivation capacity of AML1-ETO on the c-jun promoter.**

After 12 hours of growth with tetracycline (+tet) or without tetracycline (-tet), U937T and U937T/A-E cells were transiently transfected with a c-jun promoter construct ranging from bp -79 to bp +170 (pcjun -79/+170) or the empty vector pGL2 and co-transfected with 0.2 $\mu$ g of JIP-1. Cells were analyzed 24 hours post transfection. The normalized luciferase activity of each construct was arbitrarily defined as 1 fold promoter activity. The promoter activity in presence of AML1-ETO was presented in relation to it (fold promoter activity). Thus, effects of AML1-ETO on the empty vector plasmid (pGL2) could be more visible. Transfection data is shown as mean  $\pm$  S.D. of 3 separate experiments.



Figure 8B

■ cells only  
■ + JNK inh.  
■ + neg. co.



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**Figure 8B: Interfering with the JNK signaling pathway reduces AML1-ETO induced apoptosis.**

Flow cytometry analysis of AnnexinV-FITC positive U937T/A-E and U937T cells grown for 72 hours in the presence or absence of tetracycline (+tet/-tet). 1 $\mu$ M of JNK specific inhibitor peptide (JNK inh.) or negative peptide control (neg. co.) was added to the cells at the time of induction. The upper panel shows overlays of counts of AnnexinV-FITC positive cells. Cells only are represented in blue, cells + JNK inhibitor in red and cells + negative control in green. In the lower panel, the average percentages of AnnexinV-FITC positive cells were blotted against each other. Data is shown as mean  $\pm$  S.D. of three independent experiments using U937T/A-E cells and two independent experiments using U937T cells.

## 5.8 Indirect stimulation of the JNK signaling pathway by AML1-ETO: possible role for G-CSF and its receptor

The question remains how AML1-ETO, which has no known kinase activity, induced the JNK signaling pathway. To investigate if AML1-ETO indirectly stimulated JNK signaling by inducing autocrine stimuli, supernatants of U937T/A-E or U937T cells were added onto wildtype U937 cells and whole cell lysates were analyzed by Western blot for phosphorylated JNK or c-Jun. Supernatants of U937T/A-E (Figure 9A, lanes 3 and 4) but not U937T cells (Figure 9A, lanes 1 and 2) increased phosphorylation of c-Jun and JNK in wildtype U937 cells, similar to what was observed for the cellular fraction of AML1-ETO expressing cells (Figure 6B).

AML1-ETO has recently been shown to upregulate the expression of the granulocyte-colony stimulating factor (G-CSF) receptor,<sup>96</sup> which can mediate Ras-dependent activation of the JNK signaling pathway.<sup>112</sup> Furthermore, in an ovarian cancer cell line, induction with G-CSF increases JNK signaling and c-Jun expression.<sup>101</sup>

Therefore, we examined the expression of G-CSF and its receptor in U937T/A-E cells by real-time PCR and found that AML1-ETO induction increased the mRNA expression of G-CSF (Figure 9B, upper panel) and the G-CSF receptor (Figure 9B, lower panel) in the U937T/A-E cells after 24 hours of AML1-ETO induction. Furthermore, we found that priming of U937T/A-E cells after 48 hours of AML1-ETO induction with G-CSF further increased AML1-ETO induced phosphorylation of Thr183 and Tyr185 in JNK. This indicates that exogenous G-CSF enhanced the effect of AML1-ETO on JNK signaling (Figure 9C). G-CSF also slightly increased the phosphorylation of JNK in the U937T/A-E cells grown in the presence of tetracycline (U937T/A-E +tet) (Figure 9C), which might be due to the endogenous expression of the G-CSF receptor in U937 cells.<sup>113</sup>

These results indicate that AML1-ETO might stimulate JNK signaling by inducing autoregulatory loops in U937 cells, which might involve G-CSF and its receptor.

Figure 9A

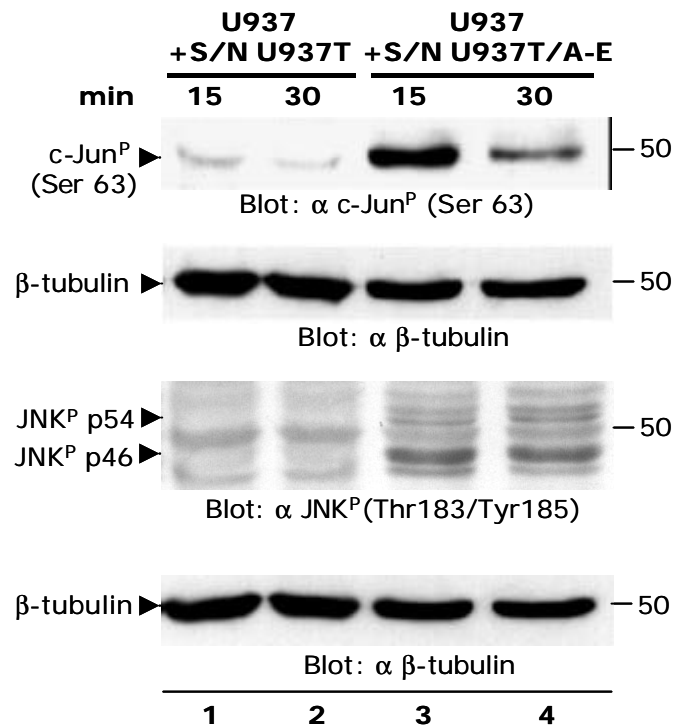
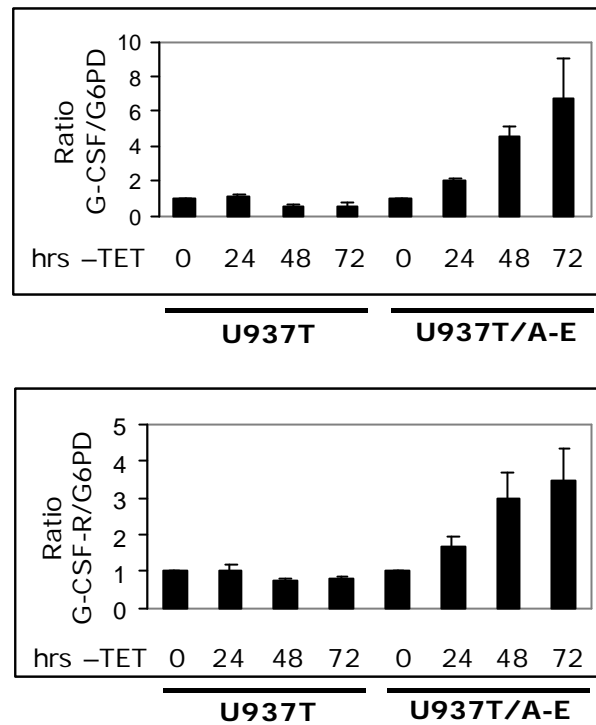


Figure 9A: Indirect activation of the JNK signaling pathway by AML1-ETO.

Western blot analysis for phosphorylated Ser63 in c-Jun: c-Jun<sup>P</sup> (Ser63) and phosphorylated Thr183 and Tyr185 in JNK: JNK<sup>P</sup> (Thr183/Tyr185). Supernatants of  $1 \times 10^7$  U937T/A-E cells (S/N U937T/A-E) (lanes 3 and 4) and U937T cells (S/N U937T) (lanes 1 and 2) were harvested 48 hours after growth in the absence of tetracycline and added to  $1 \times 10^7$  wildtype U937 cells for 15 or 30 minutes. Whole cell lysates were prepared and analyzed. Both blots were re-blotted for  $\beta$ -tubulin.

Figure 9B



**Figure 9B: Upregulation of G-CSF and G-CSF receptor mRNA expression upon AML1-ETO (A-E) induction.**

Real-time PCR for G-CSF (upper panel) and G-CSF receptor (G-CSF-R) (lower panel) of U937T/A-E and U937T cells after different hours of tetracycline withdrawal (hrs -tet). Ratios for G-CSF/G6PD (upper panel) and G-CSF-R/G6PD (lower panel) are shown as fold upregulation compared to time point zero (+tet), which was set to 1 fold. Data is shown as mean  $\pm$  S.D. of 3 different experiments.

Figure 9C

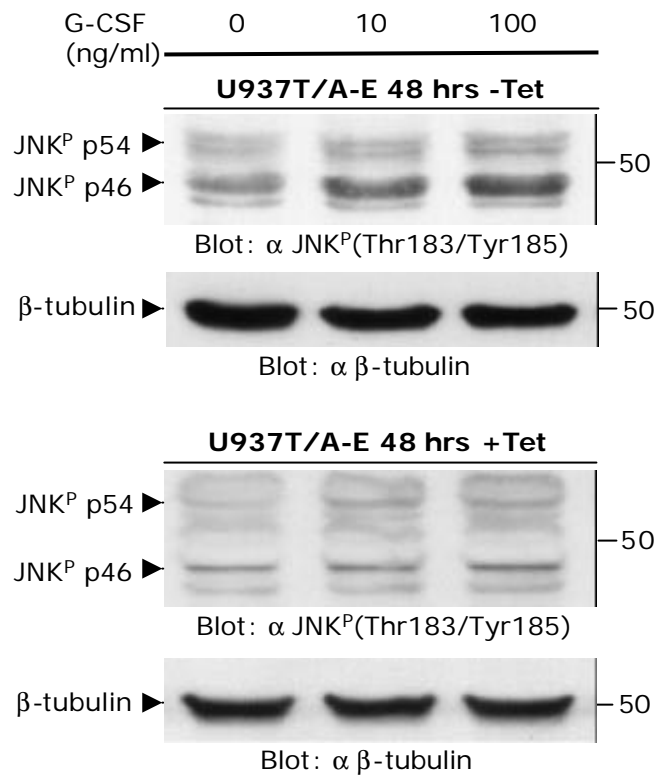


Figure 9C: G-CSF increases AML1-ETO induced phosphorylation of JNK.

Western blot for phosphorylated Thr183 and Tyr185 in JNK: JNK<sup>P</sup> (Thr183/Tyr185), detecting p46 and p54 isoforms of JNK. U937T/A-E cells were grown for 48 hours in the absence of tetracycline (48 hrs -tet) or in the presence of tetracycline (48 hrs +tet) and stimulated with 10 $\mu$ M or 100 $\mu$ M G-CSF for 15 or 30 minutes. Blots were stripped and reblotted for  $\beta$ -tubulin as loading control.

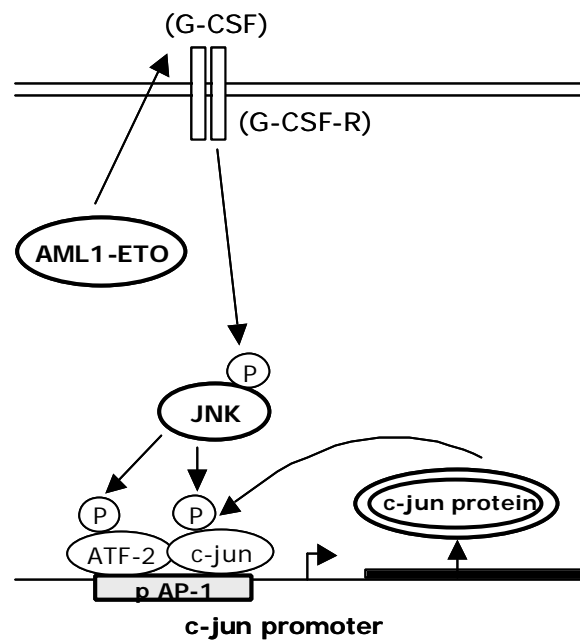


Figure 10: Model for regulation of c-Jun expression by AML1-ETO.

AML1-ETO induces c-Jun expression in an indirect manner. AML1-ETO induces the expression of a humoral factor, for example G-CSF, which in turn leads to G-CSF receptor (G-CSF-R) mediated stimulation of the JNK signaling pathway. JNK signaling connects AML1-ETO to the proximal AP-1 site of the c-jun promoter, at which pre-bound c-Jun and ATF-2 are phosphorylated by JNK. Phosphorylated c-Jun and ATF-2 stimulate c-jun promoter activity and c-Jun expression.

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## 6. DISCUSSION

Approximately 40% of AML is associated with the translocations t(8;21), t(15;17), inv(16) or t(9;11), encoding for the AML1-ETO, PML-RAR $\alpha$ , CBF $\beta$ -SMMHC, or MLL-AF9 fusion proteins, respectively.<sup>51</sup> Despite the diversity of the involved fusion partners, these AML-associated chimeric proteins deregulate their target genes through common mechanisms.<sup>114</sup> We suggest that these AML fusion proteins might also affect common targets and demonstrate in the present study that proto-oncogene c-jun was a frequently upregulated target gene (Figure 1).

In the case of t(8;21), we identified that AML1-ETO, by employing an indirect mechanism including the JNK signaling pathway, upregulated c-Jun expression through the proximal AP-1 site. Furthermore, stimulation of c-jun expression was an acquired function of the AML1-ETO chimera independent of wildtype AML1 (Figure 4B).

AML1-ETO can directly interfere with wildtype AML1 dependent transactivation in a dominant negative manner by either binding to AML1 consensus sites via the retained DNA binding domain,<sup>62,115</sup> or by physically interacting with transcription factors instead of AML1 on their target gene promoters.<sup>82-84</sup> Differential display analysis has, however, revealed that the majority of AML1-ETO target genes are independent of wildtype AML1,<sup>97</sup> which suggests that they might be regulated in an indirect manner. The c-jun promoter does not contain AML1 consensus sites and in addition, we found that wildtype AML1 had no effect on c-jun promoter activity (Figure 4B). Furthermore, AML1-ETO could not bind to the proximal AP-1 site (Figure 5), which was mapped to be the AML1-ETO responsive site (Figures 4C+D).

Physical interaction between c-Jun and AML1 has been reported via the *runt* domain of AML1,<sup>116</sup> which is retained in AML1-ETO. This suggests that prebound c-Jun might also interact with AML1-ETO and tether AML1-ETO to the promoter.



However, we could not find such interaction between prebound c-Jun and AML1-ETO at the level of the c-jun promoter (data not shown). These findings imply that regulation of the c-jun promoter by AML1-ETO is indirect.

Previous studies have indicated that specific signaling events might be important for the functional properties of AML1-ETO. Transformation capacities of AML1-ETO in NIH3T3 cells have been associated with elevated levels of phosphorylated Ser63 in c-Jun.<sup>49</sup> Also wildtype ETO requires H-Ras for transformation of 3T3 fibroblast cells.<sup>117</sup> Therefore, pathways like the JNK pathway, which are both ras and c-Jun dependent, are candidate mechanisms for AML1-ETO dependent signaling.

Unlike the leukemic fusion protein BCR-ABL, AML1-ETO has no known intrinsic kinase activity on its own, thus it appears more likely that it interferes with such signaling pathways in a rather indirect manner. ETO can interact with the regulatory subunit of type II cyclic AMP-dependent protein kinase via the NHR3 domain which is retained in AML1-ETO.<sup>118</sup> Thus, one possible mode of action might be that AML1-ETO upregulates the activity of a kinase, or downregulates the activity of a kinase phosphatase by physical binding. An alternative possibility, which we have found to be more likely, is that AML1-ETO upregulates the expression of a cytoplasmic factor, which activates receptor mediated JNK signaling. A candidate factor for this is the hematopoietic growth factor G-CSF (Figure 9).

It has previously been shown that primary AML blast cells can produce and respond to their own growth factors, like G-CSF, GM-CSF or IL1 $\beta$ ,<sup>119,120</sup> which allow them to grow autonomously.<sup>119</sup> One group found that exogenous G-CSF results in differentiation of t(8;21) positive cells<sup>121</sup> and cell lines,<sup>122</sup> and they associate G-CSF induced differentiation with activation of the STAT signaling pathway.<sup>122</sup> Stimulation of the JNK signaling pathway<sup>123</sup> and c-Jun expression<sup>101</sup> upon G-CSF administration is, however, associated with G-CSF induced growth stimulation.<sup>124,125</sup> There is also evidence that AML1-ETO might play a role in proliferation upon G-CSF. AML1-ETO blocks G-CSF induced differentiation of IL-3 dependent cell lines and leads to G-CSF dependent proliferation.<sup>87</sup>

Furthermore, AML1-ETO promotes the expansion and self-renewal capacities of human hemopoietic stem cells.<sup>89</sup> The mechanism is currently unknown, but an involvement of AML1-ETO in G1-to S-phase progression is discussed, as wildtype AML1 has shown to inhibit cell cycle progression.<sup>126</sup> Also c-Jun has been implicated in G1-to S-phase progression and cell proliferation by negatively regulating tumor suppressors and cell cycle regulators.<sup>127</sup>

We suggest that AML1-ETO and c-Jun might share additional functional properties, as c-Jun activation was also involved in the pro-apoptotic function of AML1-ETO in U937 cells (Figure 8B). As described in this study, however, inhibition of the JNK signaling pathway in U937 cells reduced apoptosis only by about 30%, suggesting additional factors within AML1-ETO induced apoptosis (Figure 8B).

This notion is further supported by our findings that AML1-ETO and c-jun mRNA expression levels correlated in primary t(8;21) positive cells and the fluctuating mRNA expression levels of AML1-ETO were responsible for the variability in c-jun expression (Figure 2). The biological relevance of the low levels of c-jun and AML1-ETO expression in some of the t(8;21) positive samples is unknown. A follow-up study on the expression levels of AML1-ETO and c-jun might give important insights whether patients with low levels of AML1-ETO and c-jun reach complete remission more rapidly.

By assuming that upregulated c-Jun expression is important for proliferation or transformation properties of leukemic blast cells, as reported in leukemic translocations in CML,<sup>44,45,47</sup> disruption of the c-Jun regulating JNK pathway might have therapeutic benefit. Inhibition of JNK has therapeutic potential in inflammatory diseases like rheumatoid arthritis<sup>128</sup> or diabetes.<sup>102</sup> t(8;21) expressing Kasumi-1 and SKNO cell lines are unique among a panel of myeloid cell lines that undergo apoptosis in response to dexamethasone treatment.<sup>129</sup> Dexamethasone, a glucocorticoid with anti-inflammatory actions, reduces c-jun mRNA levels via the glucocorticoid receptor by inhibiting transactivation via the proximal and distal AP-1 sites of the c-jun promoter.<sup>11</sup> This indicates that downregulation of c-Jun expression might be advantageous.

Alternatively, proliferation capacity in leukemic cells might be beneficial during standard chemotherapy of AML, as it increases sensitivity to S-phase specific cytotoxic agents such as cytosine arabinoside (AraC). One group investigated the proliferative activity in blast cells of various prognostic subgroups and found that blast cells from patients in the favorable subgroup including t(8;21), t(15;17) or inv(16), showed higher proliferative activity compared to the intermediate or unfavorable group.<sup>130</sup> Intriguingly, we found high levels of c-jun expression in AML patient cells with translocations associated with good response to AraC, in t(8;21), t(15;17) or inv(16), whereas c-jun expression was low in t(11q23/MLL) positive patient samples, which are associated with bad prognosis.<sup>131</sup> The good prognosis subgroups are also more likely to produce endogenous G-CSF, GM-CSF or IL3.<sup>130</sup> In accordance with this data we found that AML1-ETO upregulated the expression of G-CSF (Figure 9B, upper panel).

Besides, we found that also t(15;17) positive acute promyelocytic leukemia (APL) patient samples have high levels of c-jun mRNA in comparison to normal bone marrow cells. Interestingly, t(15;17) positive patient cells contain high levels of G-CSF receptor and proliferate upon G-CSF priming, which increases their vulnerability to cell-cycle specific drugs.<sup>132</sup> Increased endogenous G-CSF during differentiation therapy with ATRA results in proliferation,<sup>133</sup> and ATRA treatment leads to increased AP-1 activity.<sup>134</sup> These findings suggest that G-CSF, AP-1 activity and increased cell proliferation might also be important features in APL.

In summary, our results indicate that high expression levels of proto-oncogene c-jun are a common feature amongst “good prognosis” AML-associated translocations. In the case of the translocation t(8;21), AML1-ETO upregulated c-Jun expression and activity in patient cells and cell lines by positively autoregulating c-Jun expression through the proximal AP-1 site of the c-jun promoter. c-Jun regulation was indirect via the JNK signaling pathway that in turn might be indirectly stimulated by positive autoregulatory loops which might involve G-CSF (Figure 10).

We propose that the identification of common targets and mechanisms amongst “good prognosis” AML-associated translocations might provide valuable knowledge for improving the therapeutic outcome of other AMLs.

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## 7. SUMMARY

Overexpression of proto-oncogene c-jun and constitutive activation of the Jun NH<sub>2</sub>-terminal kinase (JNK) signaling pathway have been implicated in the leukemic transformation process. However, c-jun expression has not been investigated in acute myeloid leukemia (AML) cells containing the most common chromosomal translocations. t(8;21) is one of the most common AML-associated translocation and results in the AML1-ETO fusion protein. Overexpression of AML1-ETO in NIH3T3 cells leads to increased phosphorylation of Ser63 in c-Jun, which is generally JNK dependent. The role of the JNK signaling pathway for the functional properties of AML1-ETO is, however, unknown.

In the present study we found high expression levels of c-jun mRNA in t(8;21), t(15;17) or inv(16) positive patient cells by microarray analysis. Within t(8;21) positive patient samples, there was a correlation between AML1-ETO and c-jun mRNA expression levels. In myeloid U937 cells, c-jun mRNA and c-Jun protein expression levels increased upon induction of AML1-ETO. AML1-ETO transactivated the human c-jun promoter through the proximal AP-1 site via activating the JNK signaling pathway. JNK targets c-Jun and ATF-2, which also bind to the proximal AP-1 site in U937 cells, were also phosphorylated upon AML1-ETO induction. Furthermore, AML1-ETO induction increased the DNA binding capacity of c-Jun and ATF-2 to the proximal AP-1 site of the c-jun promoter, which might result in their enhanced transactivation capacities.

Interference with JNK and c-Jun activation by using JIP-1 or a JNK inhibitor reduced the transactivation capacity of AML1-ETO on the c-jun promoter and the proapoptotic function of AML1-ETO in U937 cells. AML1-ETO seems to activate the JNK signaling pathway by inducing the expression of a cytoplasmic factor, possibly G-CSF, because supernatant of AML1-ETO expressing cells was sufficient to induce phosphorylation of JNK and c-Jun in wildtype U937 cells.

This data demonstrates a novel mechanism of how AML1-ETO might exert positive effects on target gene expression and identifies the proto-oncogene c-jun as a common target gene in AML patient cells.

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## 8. ZUSAMMENFASSUNG

Überexpression des Proto-Onkogens c-jun und konstitutive Aktivierung des Jun NH<sub>2</sub>-terminalen Kinase (JNK)-Signaltransduktionsweges sind wichtig für die leukämische Transformation in der Chronischen Myeloischen Leukämie. Die Expression von c-jun bei Akuter Myeloischer Leukämie (AML) mit den häufigsten reziproken Translokationen ist jedoch unbekannt. Bei einer der häufigsten AML Translokation t(8;21) wurde in Fibroblastenzellen gezeigt, daß das AML1-ETO-Fusionsgen die Phosphorylierung des Serin 63 in c-Jun erhöht. Die Rolle des JNK-Signalweges, der c-Jun am Serin 63 phosphorylieren kann, für die Funktion von AML1-ETO wurde bisher jedoch nicht untersucht. Weiterhin kann aktiviertes c-Jun durch eine positive Rückkopplungsschleife über den c-jun Promotor zur Erhöhung der c-Jun Expression führen.

In der vorliegenden Arbeit konnten wir zeigen, daß AML Patientenzellen mit den häufigen Translokationen: t(8;21), t(15;17) oder inv(16) mehr c-jun mRNA besitzen im Vergleich zu Knochenmarkszellen gesunder Probanden. Weiterhin fanden wir eine hohe Korrelation zwischen der AML1-ETO und der c-jun mRNA bei t(8;21) positiven Patientenzellen. Induktion von AML1-ETO in der myeloischen U937 Zelllinie erhöhte sowohl c-jun mRNA als auch c-Jun Proteinexpression. Damit konnten wir zeigen, daß AML1-ETO die Erhöhung der c-jun Expression bewirkt. Wir untersuchten den molekularen Mechanismus in U937 Zellen mittels transienter Transfektionen und fanden, daß AML1-ETO den c-jun Promotor durch die proximale AP-1 Seite transaktiviert. Diese Transaktivierung erfolgte indirekt über Aktivierung des JNK-Signaltransduktionsweges durch AML1-ETO. AML1-ETO-Induktion führte auch zur Phosphorylierung der JNK-Zielproteine c-Jun und ATF-2. Diese konnten im Gelretardierungsassay an die proximale AP-1 Seite des c-jun Promotors binden und wurden durch AML1-ETO-Induktion in ihrer Bindungsfähigkeit verstärkt. Deshalb nehmen wir an, daß die Transaktivierungskapazität des c-jun Promotors durch AML1-ETO über die Aktivierung des JNK-Signalweges läuft.

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Interferenz mit der Aktivierung von JNK und c-Jun mittels JIP-1 oder eines JNK Inhibitors konnte die Transaktivierung des c-jun Promotors durch AML1-ETO und auch die pro-apoptotische Funktion von AML1-ETO in U937 Zellen reduzieren. Da AML1-ETO keine Kinasefunktion besitzt, stellt sich jedoch die Frage, wie AML1-ETO den JNK-Signalweg beeinflussen kann. Wir konnten zeigen, daß Überstände AML1-ETO exprimierender U937 Zellen ausreichend sind für die Phosphorylierung von JNK und c-Jun. Dies führte zu der Annahme, daß AML1-ETO die Expression eines cytoplasmatischen Faktors erhöht, der wiederum den JNK-Signalweg beeinflußt. Dieser Faktor könnte G-CSF sein, da wir zeigen konnten, daß die Induktion von AML1-ETO in U937 Zellen die Expression von G-CSF und des G-CSF-Rezeptors erhöht und exogene Stimulation AML1-ETO exprimierender U937 Zellen mit G-CSF die Phosphorylierung von JNK erhöht.

Die Induzierung positiver autoregulierender Loops durch AML1-ETO stellt einen neuen molekularen Mechanismus dar, wie AML1-ETO die transkriptionelle Aktivität seiner Zielgene positiv beeinflussen könnte. Weiter zeigen wir, daß das Proto-Onkogen c-jun in AML Patientenzellen mit reziproken Translokationen häufig überexprimiert ist.



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## 10. CURRICULUM VITAE

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### *PRESENTATIONS*

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#### **Talks:**

Fusion Protein AML1-ETO upregulates c-jun proto-oncogene expression in myeloid cells.  
**DGHO Mannheim 30.09.2001-3.10.2001**

#### **Poster presentations:**

Fusion Protein AML1-ETO upregulates c-jun proto-oncogene expression in myeloid cells.  
**42<sup>nd</sup> Annual Society of Hematology, San Francisco 1.-5- 2000**

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### *PUBLICATIONS*

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Gao L\*, Bellantuono I\*, **Elsasser A** Marley SB, Gordon JM, Goldman JM, and Stauss HJ.  
 "Selective Elimination of Leukemic CD34(+) Progenitor Cells by Cytotoxic T  
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 Burel S, Zhang D-E, Ueffing M, Tenen D, Hiddemann W, Behre G. "Proto-oncogene c-jun  
 expression is induced by AML1-ETO in a JNK dependent manner: possible role in the  
 pathogenesis of acute myeloid leukemia". Blood. 1<sup>st</sup> revised version submitted.



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